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# Molecular Facet of Host-Pathogen Interactions in Fusarium Head Blight in Wheat ( *Triticum Aestivum* L.)

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# MOLECULAR FACET OF HOST-PATHOGEN INTERACTIONS IN FUSARIUM HEAD BLIGHT IN WHEAT (*TRITICUM AESTIVUM* L.)

BY

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DISSERTATION ACCEPTANCE PAGE

Bimal Paudel

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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# ABBREVIATIONS

| | |
|---|---|
| **BCA** | Bicinchoninic acid |
| **BSMV** | Barley Stripe Mosaic Virus |
| **CHV1** | Cryphonectria hypovirus 1 |
| **cM** | centiMorgans |
| **CMC** | Carboxymethyl cellulose |
| **CRISPR** | Clustered Regularly Interspaced Short Palindromic Repeats |
| **CWDEs** | Cell wall degrading enzymes |
| **D3G** | Deoxynivalenol-3-glucoside |
| **dai** | days after inoculation |
| **DAPs** | Differentially accumulated proteins |
| **DEGs** | Differentially expressed genes |
| **DON** | Deoxynivalenol |
| **dpfi** | days post fungal inoculation |
| **dpvi** | days post virus inoculation |
| **eQTL** | Expression quantitative trait loci |
| **ET** | Ethylene |
| **FDK** | *Fusarium* damaged kernels |

| | |
|---|---|
| **FDR** | *Fusarium* damaged rachis |
| **FgV1** | Fusarium graminearum virus 1 |
| **FHB** | Fusarium Head Blight |
| **FRR** | Fusarium root rot |
| **hai** | hours after inoculation |
| **HCAAs** | Hydroxycinnamic acid amides |
| **hpfi** | hours post fungal inoculation |
| **HSR** | Hypersensitive reaction |
| **IWGSC** | International Wheat Genome Sequencing Consortium |
| **JA** | Jasmonic Acid |
| **kb** | kilo bases |
| **MGY** | Minimal glycerol yeast |
| **MM** | Minimum methanol |
| **NCBI** | National Center for Biotechnology Information |
| **NIL-R** | Near isogenic line-Resistant |
| **NILs** | Near isogenic lines |
| **NIL-S** | Near isogenic line-susceptible |
| **ORF** | Open reading frame |

| | |
|---|---|
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PCR** | Polymerase chain reaction |
| **PDA** | Potato dextrose agar |
| **PDB** | Potato dextrose broth |
| **PMEI** | Pectin methyl esterase inhibitor |
| **qPCR** | quantitative polymerase chain reaction |
| **QTL** | Quantitative Trait Loci |
| **RACE** | Rapid amplification of cDNA ends |
| **RT** | Room temperature |
| **RT-PCR** | Reverse transcription polymerase chain reaction |
| **RT-qPCR** | Reverse transcription quantitative polymerase chain reaction |
| **siRNA** | small RNAs |
| **SSR** | Simple Sequence Repeat |
| **VIGS** | Virus induced gene silencing |
| **VOX** | Virus-mediated overexpression |
| **YPD** | Yeast extract peptone dextrose |

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# ABSTRACT

MOLECULAR FACET OF HOST-PATHOGEN INTERACTIONS IN FUSARIUM HEAD BLIGHT IN WHEAT (*TRITICUM AESTIVUM* L.)

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Fusarium head blight (FHB) is a severe disease of wheat (*Triticum aestivum* L.). It not only reduces the quantity of the harvested grains but also decreases the grain quality due to mycotoxins contamination, especially Deoxynivalenol (DON). *Qfhb1* (or simply called *Fhb1*) is the most important quantitative trait locus (QTL) for FHB resistance. Our lab has previously identified wheat gene *WFhb1-1* (aka. *Wfhb1_c1*) as a candidate for FHB resistance gene. Here we report that *WFhb1-1* has been cloned. The gene (GenBank # KU304333.1) consists of a single exon, encoding a putative membrane protein of 127 amino acids. WFhb1-1 protein produced in *Pichia pastoris* inhibits the growth of both *F. graminearum* and *P. pastoris* in culture. Western Blotting with anti-WFhb1-1 antibody revealed a significant decrease ($p < 0.01$) in WFhb1-1 accumulation 12 hours post *Fusarium* inoculation in *Qfhb1*-non carrier wheat but not in *Qfhb1*-carrier wheat. Overexpressing *WFhb1-1* in *Qfhb1*-non carrier wheat led to a significant decrease ($p < 0.01$) in *Fusarium*-damaged rachis rate, *Fusarium*-diseased kernel rate and DON content in harvested kernels, while silencing *WFhb1-1* in *Qfhb1*-carrier wheat resulted in a significant increase ($p < 0.01$) in FHB severity. Therefore, *WFhb1-1* is an important FHB resistance gene with a potential antifungal function and probably a key functional component of *Qfhb1* in wheat. A model regarding how *WFhb1-1* functions in FHB

resistance/susceptibility is hypothesized and discussed. All the published reports on *Fhb1* candidate genes has been also reviewed, discussed, and a multi-gene model of *Qfhb1* associated regulation mechanism against FHB has been proposed.

DON toxin production during the infection of *F. graminearum* in small grain crops is one of the most harmful virulence factors associated with economic losses. *F. graminearum* strain, Fg-4-2, having mycovirus FgV1-SD4 showed significantly reduced virulence against wheat compared to the wildtype not having the virus. Additionally, no DON accumulation was detected in the harvested wheat seeds infected by Fg-4-2, whereas ~18 ppm DON was detected in seeds infected by Fg-4-1, (*F. graminearum* not having FgV1-SD4). In this study, we propose a pathway of regulation of DON biosynthesis by FgV1 infection. We also made an infectious clone of the mycovirus, FgV1-SD4, and tested if this strain of FgV1 can infect *F. graminearum* and *F. oxysporum* by electroporation method of transfection. Although, we could not confirm the transfection of *F. oxysporum* and *F. graminearum*, differences in culture morphology and pathogenicity were observed between the transfected and the wildtype strains. If this mycovirus strain and the protocol of transfection can be optimized to infect broad range of *F. graminearum* and *F. oxysporum* strains, it can open a possibility of using this FgV1 strain as a potential biocontrol agent against *F. graminearum* infections to reduce the DON accumulation level in small grain crops due to this fungal disease.

A protocol for the overexpression of *WFhb1-1* gene using BSMV virus-mediated over-expression (VOX) is also developed. Using a three plasmid BSMV expression system, up to ~30-fold overexpression of *WFhb1-1* gene could be achieved in generation T0 of wheat plants, whereas we could detect ~2.5-fold overexpression of the gene in T1

generation transmitted through seeds into the new generation. As far as we know, this is the first report of transmission BSMV-mediated gene overexpression through seeds into the new generation. This has a huge potential for further exploration in the use of BSMV in overexpression of genes in many generations, and optimization of the process for gene editing using CRISPR/Cas9 technique.

# Chapter 1

## Literature review

### 1.1 FHB and its economic impacts

Fusarium head blight (FHB), or head scab or scab is one of the most damaging diseases of cereal grains such as wheat and barley. The disease is so severe that it can cause significant yield losses within a few weeks before harvest (McMullen et al., 1997). Reduced yields, shriveled grains, mycotoxin contamination, and reduction in seed quality are major factors that are related to losses due to this disease (Parry et al., 1995). Infected grains may get contaminated with different mycotoxins, for instance deoxynivalenol (DON) and other heat stable trichothecene type B toxins. These toxins remain in processed foods and cause health hazards in humans and animals (Pestka, 2010). DON is the most prevalent and serious toxin produced by *F. graminearum* during its infection in wheat. The United States, the European Union, and many other countries have DON regulation policies that dictate maximum levels allowed in processed foods (European Union law, 2006; U.S. Food & Drug Administration, 2010). Therefore, the grains harvested during FHB epidemics lose market, thus causing high economic burdens to the farmers.

Since its first description in England in 1884, FHB has been a disease difficult to control and forecast (Stack, 2000). FHB epidemics has been recorded as early as 1800s (Adams, 1921). Many FHB epidemics were recorded in 1900s in 26 states of the USA and four provinces in Canada (Quebec, Ontario, Manitoba and Saskatchewan) and significant economic losses due to FHB have been widely reported since the 1950s in the

USA (Boosalis et al., 1983; Gilbert and Haber, 2013; McMullen et al., 1997; Parry et al., 1995; Windels, 2000). In 1982, scab caused an estimated 4% reduction in the total United States wheat production. Similarly, the soft red winter wheat areas of the Midwest, Southeast, and Mid-Atlantic states endured loss of harvest of 2.7 million metric tons in 1991. The scab epidemic of South Dakota, North Dakota, and Minnesota in 1993 was so severe that a loss of $1 billion was estimated (Nganje et al., 2004). Additionally, over $2.7 billion in economic loss has been reported between 1998 and 2002 in the Northern and Central USA (Nganje et al., 2002). Although progress has been made in controlling scab epidemics, success has been limited, and wide economic losses due to this disease are still reported (Cowger and Sutton, 2005). Therefore, FHB still persists as an epidemic threat in different states, thus posing a great risk of economic losses due to reduction in small grain harvests and toxins contamination.

**1.2 FHB disease development, causative agent, and its symptoms**

FHB is a disease of primarily small grain crops like wheat, barley and oat, but maize, rice and other small grain crops can also be infected. FHB can caused by many species of *Fusarium*; however, *F. graminearum* Schwabe is the primary pathogen of FHB. This is also called *Gibberella zeae* Schw. (Peth) during its sexual stage of life cycle; however, this name is rarely used lately (Stack, 2003). Other species that can cause FHB are *F. avenaceum, F. culmorum, F. poae, F. cerealis, F. equiseti, F. sporotrichoides, F. tricinctum, F. acuminatum, F. subglutinans, F. solani, F. oxysporum, F. verticillioides, F. semitectum, and F. proliferatum* (Liddell, 2003). FHB is seen as a head bleach in wheat, which could be confused with other diseases that also cause bleaching of heads. Head bleach in FHB is slightly darkened and oily areas on infected

florets when the environment is warm and humid. The spike usually shows a pinkish color in rachis and glumes due to production of conidiospores in sporodochia. Sometimes, growth of *F. graminearum* mycelia may give white color to spikes (Monreno-sevilla, 2013). Additionally, developing kernels become shrunken and wrinkled in FHB due to fungal colonization.

### 1.3 Pathogenicity of *F. graminearum*

*F. graminearum* population has been broadly categorized into two groups. First group is characterized by the isolates that do not form perithecia in culture or in natural conditions. Second group is characterized by the isolates that produce perithecia in the spring by surviving as saprophytic organism with mycelium growth in the remains of wheat and corn over the winter (Parry et al., 1995; Francis and Burgess, 1997). These pathogenic perithecia can be propagated by airborne inoculum and cause the disease. *F. graminearum* isolates causing FHB belong to the second group. Therefore, there are two phages in the life cycle of pathogenic strains of *F. graminearum*: saprophytic phase and pathogenic phase.

Bushnell et al. (2003) have comprehensively reviewed the pathogenesis of *F. graminearum*. Inoculum, environmental conditions, and initial infections are the most important factors in disease development. Pathogenic phase of FHB starts when spores, macroconidia and other propagules are carried away by air or splashing water and land on or inside spike tissue during anthesis (Bushnell et al., 2003; Goswami and Kistler, 2004). Temperature and humidity during the flowering stage play crucial role in initial disease development. Similarly, soil conditions, such as nitrogen fertilization, plant density, competition of *Fusarium* with other microorganisms also influence disease dynamics of

FHB (Osborne and Stein, 2007). However, these environmental factors are interdependent on each other during disease development. Optimal temperature and continued moisture result in more pronounced growth of the pathogen (Anderson, 1948; Pugh et al., 1933). Water potential of -1 to -20 bars is reported to be optimal for spore germination and when it falls below -60 the germination is completely inhibited (Colhoun et al., 1968; Sung and Cook, 1981).

*F. graminearum* cannot initially penetrate the thick-walled epidermal cells on the exterior surfaces of spikes, but it develops mycelia on outer surfaces after germination (Kang and Buchenauer, 2000; Bushnell et al., 2003). Growth of network of mycelium on exterior surfaces makes it possible for fungus to reach to stomata, crevices between the palea and lemma, and anthers making its way into interior structures, and sometimes actively by direct penetration of the inflorescence facilitated by various cell wall degrading enzymes (Bushnell et al., 2003; Lewandowski and Bushnell, 2001; Walter et al., 2010). After penetration, the pathogen grows intracellularly and spreads through xylem and pith. This stage of infection is known as biotrophic phase of infection, which is followed by second phase of infection known as necrotrophic phase (Guenther and Trail, 2005; Seong et al., 2008). The transition from biotrophic phase to necrotrophic phase is mediated in part by mycotoxins primarily deoxynivalenol (DON) (Bushnell et al., 2003). At necrotrophic stage of infection, rapid colonization of the pathogen occurs, and it spreads to new adjacent florets rapidly through vascular bundles in rachis and rachilla (Ribichich et al., 2000). Through this mechanism, the pathogen spreads from spikelet to spikelet. Due to rapid growth of the pathogen in all surrounding tissues and mycotoxin production, vascular bundles in infected rachis become dysfunctional and

drying and bleaching of spikelets is observed leading to all FHB symptoms. Necrotrophic phase of growth is accompanied with mycotoxins accumulation and damaged kernels resulting in shriveled, undersized grains known as tombstones (Bushnell et al., 2003; Harris et al., 1999).

Next phase of *F. graminearum* life cycle is saprophytic phase of growth after the crop is harvested and death of plants (Sutton, 1982). *F. graminearum* produces many hydrolyzing enzymes and colonizes crop residues to survive over many years in soil. When the conditions are favorable ascospores are produced from developed perithecia and start new cycle of infection in new season.

### 1.4 FHB management

The severity of FHB is controlled mainly by the following factors: (i) genetic factors of the host, (ii) genetic factors of the pathogen, and (iii) environmental influences (Buerstmayr et al., 2009; Campbell and Lipps, 1998; Fuentes et al., 2005). Cultivating resistant cultivars is the most effective approach to minimize losses associated with FHB in wheat, barley, and other small grain crops caused by *F. graminearum* (McMullen et al., 1997). Economic losses due to FHB are huge; however, highly resistant cultivars against FHB are not available. Sources used by breeders to develop resistant cultivars are not abundant and available sources do not contribute for high level of resistance. Therefore, multiple approaches should be combined to manage FHB effectively. Some of the strategies that can help to manage FHB are improving cultivar resistance, fungicide application, development and use of biological control agents. Similarly, crop rotation, tillage operation, and FHB forecasting would also help in its management (McMullen et

al., 1997; McMullen et al., 2012).

### 1.4.1 FHB resistance

FHB resistance is a complex trait, and two types of FHB resistance are widely accepted. Resistance to the initial infection is considered as type I, whereas resistance to the spread of the infection within the head is considered as type II (Schroeder and Christensen, 1963). Mechanisms underlying type I resistance are unclear but likely contributed by spike morphology, and activation of systemic innate immune response in the host (Foroud et al., 2012; Mesterhazy, 1995). Type II resistance is attributed by different resistant genes and has been more extensively studied. The application of type II resistance has more scope with its better utilization in development of resistant cultivars. Multiple genetic studies using various resistant wheat cultivars have revealed that type II resistance is supposedly controlled by two to three major genes and a few minor genes (Buerstmayr et al., 2009; Gao et al., 2005; Zhuang et al., 2013).

### 1.4.2 FHB resistance QTLs

Multiple quantitative trait loci (QTL) studies have been carried out for the FHB resistance. Over 100 QTLs has been identified in all the wheat chromosomes associated with resistance/susceptibility against FHB (Gosman et al., 2008; Buerstmayr et al., 2009). However, most of the reported QTLs either cannot be verified in different genetic backgrounds or contribute none to very small part of FHB resistance/susceptibility. There are very few QTLs that contribute relatively high percentage of FHB resistance. *Qfhs.ndsu.3BS* (also called *Qfhb1* or *Fhb1*) and *Qfhs.ifa-5A* are the two major QTLs that contribute most significant FHB resistance in different genetic backgrounds. *Qfhb1* is

known to contribute Type II resistance against pathogen spreading and mycotoxin degradation, especially DON, whereas *Qfhs.ifa-5A* is known to contribute resistance against initial infection (Anderson et al., 2001; Buerstmayr et al., 2003; Lemmens et al., 2005; Liu et al., 2006; Buerstmayr et al., 2009). Few other QTLs, namely *Qfhb2*, *Qfhb3* and *Qfhb4* were also claimed to provide high percentage of FHB resistance in certain mapping populations; however, these claims remain unfounded because the contributed resistance by these QTLs in other genetic backgrounds is very little (Cutbert et al., 2007; Qi et al., 2008; Xue et al., 2010).

### 1.4.3 QTL *Qfhb1*

QTL *Qfhb1*, originally identified in Chinese cultivar Sumai3, is known to provide a moderate level of genetic resistance against FHB. *Qfhb1* could account up to 60% of the total phenotypic variation in FHB resistance (Buerstmayr et al., 2009) and is the most effective, and most stable QTL across different genetic backgrounds, and accounts for most of the FHB resistance (Anderson et al., 2001; Basnet et al., 2012; Somers et al., 2003; Zhou et al., 2002). Sumai3 and other cultivars derived from it, such as 'Ning 7840', 'Frontana' from Brazil, 'Nobeokar' from Japan and 'Freedom' in eastern soft red winter wheat region of the USA, are used as best sources to breed FHB resistant cultivars worldwide for more than a half century (Dubin, 1997; Wilde et al., 2007). *Qfhb1* is located on chromosome arm 3BS, and is flanked by two simple sequence repeat (SSR) marker loci, *Xgwm533* and *Xgwm493,* and was later verified in many wheat populations (Anderson et al., 2001; Bai et al., 1999; Buerstmayr et al., 2009; Liu et al., 2006; Zhou et al., 2002). Liu et al. (2008) narrowed the QTL region down to 1.2cM interval between markers sts3B-189 and sts3B-206, which was found to host seven genes in Chinese

Spring and 13 genes in Sumai 3 (Liu et al., 2008; Rawat et al., 2016). However, the exact region and sequence encompassed by *Qfhb1* is still controversial, which might partly be due to dissimilarities in the sequence between different wheat accessions. In fact, Schweiger et al. (2016) reported a high dissimilarity between Sumai3 and Chinese Spring in the core region of *Qfhb1* that hosts the FHB marker UMN10, and three published *Qfhb1* candidate genes: *TaGDSL* (Schweiger et al., 2016), *TaPFT* (Rawat et al., 2016) and *TaHRC* (Su et al., 2018; Su et al., 2019). Moreover, almost all-important markers associated with this QTL have inconsistent positions on chromosome 3BS among different wheat accessions used in different mapping studies, which indicates inversions between different wheat accessions. For example, marker *Xbarc147* is located at proximal side of marker *Xgwm533* in the Sumai3/Stoa map by Liu et al. (2006) but located at the distal side of *Xgwm533* in the Wangshuibai/Wheaton map by Yu et al. (2008). Similarly, the marker *Xgwm493* is moved from the distal side of *Xumn10* in the Chinese Spring 3B pseudomolecule (Fig 1) to proximal side in Sumai3/Stoa (Liu et al., 2006). Additionally, marker *Xbarc147* is at the distal side of *Wfhb1_c1* (aka *WFhb1-1*) in Sumai3, as reported by Zhuang et al. (2013), and similarly, at the distal side of *Qfhb1* interval in Sumai3/Stoa (Liu et al, 2006) but in the middle of *Qfhb1* in the Wangshuibai/Wheaton map by Yu et al. (2008). Interestingly, *TaPFT* gene reported by Rawat et al. (2016) as a candidate gene for *Qfhb1* was reported to lie outside of the QTL in FHB-resistant Wangshuibai by Li et al. (2019). Therefore, the exact location of *Qfhb1* is not so clear and debate about it is still going on.

Schweiger et al. (2016) sequenced 1029 kb contig that encompasses *Qfhb1* (GenBank accession: KU641029) from CM82036, a highly FHB resistant Sumai-3

derivative line (*Qfhb1*-carrier). They also compared this sequence with the same region in the reference genome of susceptible line, Chinese spring, and found that it includes a 395 kb highly dissimilar sequence. They identified 33 genes in the contig, localized the *Qfhb1* in a region of 860 kb harboring 28 genes using fine mapping of identified recombinant lines with cross-over events to the sequenced contig. Interestingly, this study found that this contig was hard to crossover between the FHB-resistant and the FHB-susceptible wheat lines, probably due to large sequence dissimilarity. This sequence dissimilarity is also embodied in quite a few other QTL mapping results (Fig. 1.1). Clearly, gene linkage of this QTL may be different between wheat genotypes.

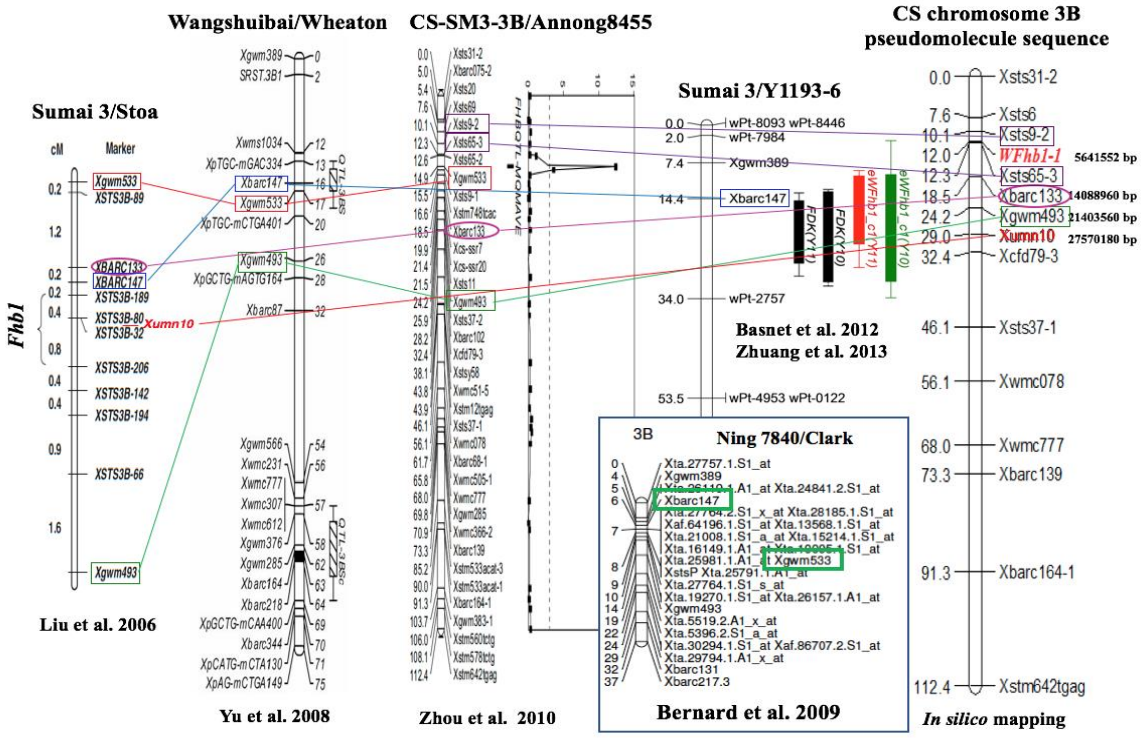


**Fig 1.1.** Mapping of *Qfhb1* by different groups of researchers.

### 1.4.4 Functional mechanism of *Qfhb1*

Many studies to isolate a gene associated with *Qfhb1* have been challenging with

inconclusive and contradictory outcomes. Additionally, sequenced genome of the wheat chromosome 3BS of the cultivar Chinese Spring (Choulet et al., 2010), which is an FHB susceptible line, has not shed much light either on the underlying genetic determinants of *Qfhb1*. Different studies have proposed different candidate genes and different roles of *Qfhb1* (Table 1.1).

There have been a few transcriptomics and proteomics study comparing differentially expressed genes (DEGs) or differentially accumulated proteins (DAPs) between the lines carrying and non-carrying *Qfhb1* (Eldakak et al., 2018; Hofstad et al., 2016; Gunnaiah et al., 2012). At the transcriptomic level, Hofstad et al. (2016) identified genes that were expressed in the *Qfhb1*-carrying genotype but not in *Qfhb1*-noncarrying genotype, and found three genes out of 12 DEGs that were upregulated in the former genotype compared to the later. Based on this study, a receptor protein kinase and the chaperone protein DnaJ were mapped to 3BS chromosome, among these three genes identified in *Qfhb1*-carrying genotype. MYB transcription factor was the third gene that did not map to *Qfhb1* region. Additionally, critical role of rachis cells in FHB resistance is revealed by these studies (Hofstad et al., 2016; Gunnaiah et al., 2012).

Eldakak et al. (2018) conclude in their quantitative proteomic study that *Qfhb1* confers resistance in wheat cultivars carrying the QTL by manipulating the cells to not respond to the *Fusarium* infection for the defense by alleviating hypersensitive response (HSR). They found that the susceptibility related genes were upregulated in *Qfhb1*-non carrying wheat lines compared to the *Qfhb1*-carrying wheat lines and *Qfhb1* confers resistance by not responding to the pathogen mediated HSR response in resistant wheat lines.

Earlier works on the role of *Qfhb1* have reported that it contributes to resistance against DON (Lemmens et al., 2005). Other groups of researchers who further pursued this line of research on the role of *Qfhb1* have reported that *Qfhb1* plays a role in the breakdown of DON to DON-3-O-glucoside (D3G) via uridine diphosphate-glycosyltransferase (UDP-glycosyltransferase or *UGT*) (Gardiner et al., 2010; Schweiger et al., 2010; Schweiger et al., 2013). *Qfhb1* has also been reported to mediate resistance by high levels of phenylpropanoids, which might also be related to the higher level of Jasmonic acid, a biotic stress hormone (Gunnaiah et al., 2014). Alternatively, Zhuang et al. (2013) suggested that a putative pectin methyl esterase inhibitor gene (PMEI) could be associated to *Qfhb1* because of the downregulation of the expression of this gene immediately after pathogen infection in wheat lines that lack *Qfhb1*. In contrast, Rawat et al. (2016) associated a pore-forming toxin-like (*TaPFT*) gene with *Qfhb1* for FHB resistance using map-based cloning. More recently, Su et al., (2019) and Li et al., (2019) independently concluded that deletion mutation of His-rich Ca-binding protein is associated with *Qfhb1* mediated FHB resistance.

#### 1.4.4.1 *Qfhb1 against DON*

DON, which belongs to trichothecenes family of toxins, is not only a mycotoxin that contaminate harvested grains, but also a virulence factor for the *F. graminearum* in FHB development. Trichothecenes impact the growth of host cell growth by inhibiting protein biosynthesis (Gunupuru et al., 2017). DON binds 60S subunit of ribosome and inhibits either the chain initiation, elongation or termination during protein synthesis. Consequently, the overall effects of DON to the host cells include lipid peroxidation, apoptosis, ribotoxic stresss, DNA synthesis inhibition, disruption of cell membrane and

suppression of cell division (as reviewed in Arunachalam and Doohan, 2013). All of these factors aid the pathogen to establish disease in FHB, especially during necrotrophic phase of the disease cycle.

**Table 1.1.** Genes associated with *Qfhb1*

| **Gene annotation** | **Gene** | **Reference** |
|---|---|---|
| UDP glucosyltransferase | *TaUGT3* | (Lulin et al., 2010; Schweiger et al., 2010) |
| Pectin methyl esterase inhibitor | *Wfhb1_c1* (aka *WFhb1-1*) | (Zhuang et al., 2013; Paudel et al., 2017) |
| Pore-forming toxin-like | *TaPFT* | (Rawat et al., 2016) |
| GDSL Lipase | *TaGDSL* | (Schweiger et al., 2016) |
| His-rich Ca-binding protein | *TaHRC* or *TaHis* | (Su et al., 2019; Li et al., 2019) |

*Qfhb1* has been noticed to be associated with low DON content since it was revealed. To substantiate this association, Lemmens et al. (2005) conducted a genetic study of DON metabolism in wheat by directly applying DON to the flowering wheat spikes with or without *Qfhb1*. They found that the ratio of D3G:DON was significantly higher in resistant lines having *Qfhb1* compared to susceptible lines that lack the QTL. UDP-glycosyltransferase, which can convert DON to D3G, has been reported as a major enzyme associated with this process in barley (Schweiger et al., 2013). It is also shown that the overexpression of barley UDP-glucosyltransferase in *A. thaliana* has enhanced resistance against DON (Poppenberger et al., 2003; Shin et al., 2012).

Similarly, Schweiger et al., (2010) reported higher rate of breakdown of DON by heterologous expression of UDP-glucosyltransferase from barley in yeast compared to the untransformed yeast (Schweiger et al., 2010). Specific UDP-glycosyltransferase named as *TaUGT3*, a DON-induced wheat *UGT* gene, is located in the 3BS region of resistant cultivar Wangshibai (Lulin et al., 2010), but it is not specifically located within the *Qfhb1* QTL, and two other copies of the gene in chromosome 3AS and 3DS were also been reported in the same study. This study also reported DON-resistance function of the gene by overexpressing *TaUGT3* in *A. thaliana* and showed less growth inhibition of transformants by DON than untransformed controls. However, cloning of wheat *TaUGT3* cDNA into yeast expression vector did not confer any resistance against DON when the transformed yeast was grown in YPD plates containing DON, and compared to the untransformed control (Schweiger et al., 2010). Moreover, recent sequencing of the contig (nearly 860 kb) that localizes *Qfhb1* failed to show any *UGT* gene inside the contig (Schweiger et al., 2016; Barbara et al., 2017). Now, studies are ongoing beyond the region covered by the contig mentioned above to reveal any *UGT* gene associated with *Qfhb1* (Barbara et al., 2017). Moreover, another study has contrasted the idea whether the degradation of DON is the absolute indicator of FHB resistance or not. This study has reported a higher D3G:DON ratio in highly susceptible lines compared to resistant lines using CIMMYT wheat elite germplasm with various levels of FHB resistance (Nakagawa et al., 2017). This suggests that DON resistance and FHB resistance might be two different concepts, and possibly regulated by separate mechanisms. DON resistance via its breakdown to D3G might be just a part of the FHB resistance in some cases, but not an indicator of FHB resistance in itself. Therefore, DON

breakdown might not necessarily predict overall FHB resistance.

#### 1.4.4.2 *Qfhb1 and phenylpropanoids*

Increased level of expression of polypropanoids in *Qfhb1*-carrier wheat lines compared to *Qfhb1*-non carrier lines was proposed by Gunnaiah et al. (2012) based on their metabolomo-proteomics study. They associated *Qfhb1* with cell wall thickening and breakdown of DON to D3G by using near-isogenic lines of wheat having or not having *Qfhb1*. In their study, strengthening of rachis cell walls through deposition of hydroxycinnamic acid amides (HCAAs), flavonoids and phenolic glycosides that are components of the phenylpropanoid metabolism, was proposed as a resistance factor in wheat lines having *Qfhb1*. Similarly, they also showed upregulation of enzymes involved in methionine metabolism in *Qfhb1*-carrier wheat lines, which also tilts the metabolic flux towards the phenylpropanoid pathway. *Qfhb1* is also physically mapped in the contig ctg0954 that carries 41 genes (Choulet et al., 2010), and Gunnaiah et al. (2014) predicted a functional domain of N-hydroxycinnamoyl/benzyltransferase in this contig that is involved in HCAA biosynthesis. Seven genes that encode different classes of glycosyltransferases and one gene coding for methyltransferase containing protein were also located in the same contig ctg0954 (Choulet et al., 2010), but the specific role of these genes and their association with *Qfhb1* mediated resistance remains unknown.

Li and Yen (2008) associated FHB resistance with JA and ET pathways, reporting that the spraying of JA and ET before *F. graminearum* inoculation changed highly susceptible wheat cultivar Y1193-6 to be resistant comparably to Sumai 3. A possible role of JA in type II FHB resistance in rachises was also reported by Gunnaiah et al. (2012). They reported higher accumulation of JA and its precursors in NIL-R genotype

compared to NIL-S genotype, having, or not having *Qfhb1*. They also proposed that DON induces ET and JA signaling in NIL-R wheat lines, which then upregulates HCAAs biosynthesis. Therefore, the cell wall thickening in NIL-R lines, they found, was associated with JA and ET pathways that signal towards upregulation of HCAAs. However, it is not clear how the *Qfhb1* plays a role in JA and ET pathways and cell wall thickening in NIL-R (*Qfhb1*-carrier) lines compared to NIL-S (*Qfhb1*-non carrier) lines.

#### 1.4.4.3 *Qfhb1 and putative pectin methyl esterase inhibitor*

A functional study associating transcript abundance to genetically mapped positions in an eQTL study was carried out by Zhuang et al. (2013). Using combined efforts with transcriptome analysis, genetic studies, eQTL assay, and physical mapping, they identified a functional gene, temporarily named *Wfhb1-c1* (*wheat Fhb1 candidate gene -1*) associated with *Qfhb1*. This gene could explain 24 - 39% of the phenotypic variation in FHB during their two-year study period. The *Wfhb1-c1* gene is present both in resistant and susceptible wheat genotypes regardless of the presence or absence of *Qfhb1*. However, in susceptible lines not having *Qfhb1*, such as Y1193-6 and NIL-S, the expression of this gene was suppressed over 20-fold following immediately after inoculation of the pathogen (Fig 2). In contrary, this suppression of *Wfhb1-c1* gene expression was not observed in Sumai-3 and NIL-R (*Qfhb1*-carrier). Therefore, there might be a regulation mechanism either through another wheat gene or through a fungal gene, which suppresses expression of the *Wfhb1-c1* gene immediately after infection in susceptible wheat lines. Wang et al. (2018) independently verified the role of *Wfhb1-c1* not only against FHB but also against Fusarium root rot (FRR) by prevention of the pathogen from its spreading in the infected host plant.

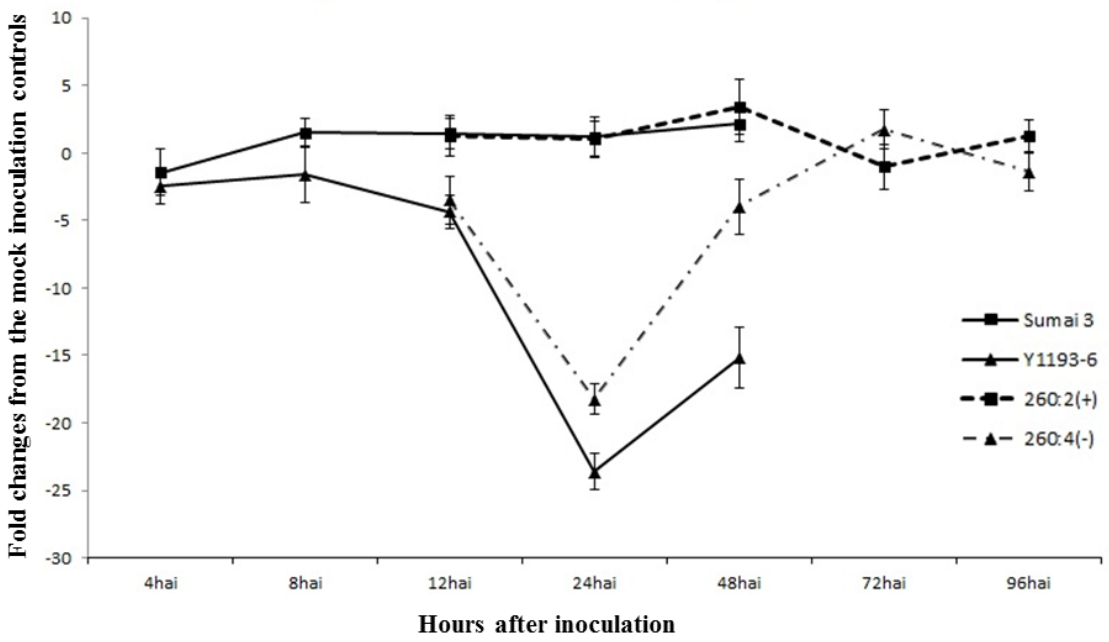


**Fig 1.2.** Expression Changes of Wheat Gene *WFhb1-1* (aka *Wfhb1-c1)* in the Inoculated Spikelets of FHB-resistant Sumai 3 and NIL-260-2, and of FHB-susceptible Y1193-6 and NIL-260-4 after Infection by *F. graminearum* (Reprinted with permission from Zhuang et. al., 2013).

Although the specific function of *Wfhb1-c1* protein in a cell is not yet known, Zhuang et al. (2013) suggested that it could be a PMEI gene that is involved in cell wall modification. PMEI genes and the process of cell wall modification is widely reported to be involved in resistance against necrotrophic pathogens including *Fusarium* in different plants (An et al., 2009; An et al., 2008; Lionetti et al., 2012; Volpi et al., 2011). Although this gene is functionally associated to *Qfhb1*, the gene is not located in the 1-Mb contig that encompasses *Qfhb1*, sequenced by Schweiger et al. (2016).

#### 1.4.4.4 *Qfhb1 and GDSL lipase*

Schweiger et al. (2016) observed that a gene that encodes *GDSL lipase* was the only gene present in the *Qfhb1*-carrier line that showed a significant increase in its

expression level in response to pathogen inoculation. GDSL lipase/esterases occur as diverse gene families across a wide range of plants. For example, 114 members exist in rice (Chepyshko et al., 2012). They are known to act in defense mechanisms in plants along with various other physiological functions. *GDSL lipase-1* in *A. thaliana* is known to mediate innate immunity through ET signaling pathway against necrotrophic pathogens (Kim et al., 2014; Kwon et al., 2009). However, this *GDSL lipase-1* does not share sequence similarity with *GDSL lipase* found in the contig KU641029 harboring *Qfhb1* in wheat (*TaGDSL*) (Schweiger et al., 2016). No further studies on silencing or overexpression of this *TaGDSL*, or how the gene is regulated in *Qfhb1* region have been reported. Therefore, the functional association of *TaGDSL* with FHB resistant trait remains largely unknown. However, Rawat et al. (2016) showed that *TaGDSL* gene was indifferently expressed in both the *Qfhb1*-carrier and *Qfhb1*-non carrier lines in their study. More recent reports suggest that the *TaGDSL* has also been found in different wheat accessions from many countries where *Qfhb1* has never been detected (Su et al., 2019; Li et al., 2019).

#### 1.4.4.5 *Qfhb1 and pore-forming toxin-like protein*

Rawat et al., (2016) suggested a pore-forming toxin-like protein (*TaPFT*) that is associated with *Qfhb1* and plays a role against FHB. They used a combination of mutation analysis, gene sequencing and transgenic overexpression of this gene to conclude this finding. This gene encodes a pore-forming toxin-like protein with chimeric lectin and ETX/MTX2 toxin domain. Surprisingly, they could not find any role of this gene in DON detoxification. Revealing of *TaPFT* gene was considered as a major breakthrough among wheat research community when the report was published;

however, this promise did not last long. Schweiger et al. (2016) reported that no expression difference of this gene was observed between the FHB-inoculated and mock control, nor between the NILs with or without *Qfhb1*, and that no inhibition to yeast growth by this gene was observed when it was transformed into the yeast. Moreover, Su et al. (2019) and Li et al. (2019) reported that the *PFT* gene is present even in susceptible wheat lines that completely lack *Qfhb1* and based on the later report this gene lies outside of *Qfhb1* (Li et al., 2019).

#### 1.4.4.6 *Qfhb1 and His-rich Ca-binding protein*

Su et al. (2019) and Li et al. (2019) independently proposed a new gene associated with *Qfhb1*, which is annotated as His-rich Ca-binding protein (*TaHRC* by former and *TaHis* by later). This gene has 2,650 bp sequence and a deletion spanning the start codon of this gene results in FHB resistance. This deletion makes this susceptible gene non-functional thus conferring resistance in deletion mutants (Su et al., 2017; Su et al., 2019). However, Li et al. (2019) claim that the deletion makes frameshift mutation and encodes different proteins for FHB resistance, and they named the resistant gene as $His^R$. A deletion of 752 bp including 24 bp 5' sequence in $His^R$, compared to $His^S$, of the ORF causes loss of the 3' splicing acceptor site and generation of new acceptor site, which results in change of translation codon (Li et al., 2019). The resulted $His^R$ polypeptide is 14 residues longer and different from $His^s$ by 21 amino (N)-terminal residues.

On the contrary, Su et al. (2019) did an association mapping panel, sequencing analysis, RNA interference and gene editing for FHB resistance using resistant haplotype Ning 7840 and susceptible haplotype Clark and Chokwang, and found that the loss of

function of *TaHRC* significantly increases FHB resistance in susceptible lines. They also did a screening of 1,632 wheat landraces and cultivars from 73 different countries and found that the *TaPFT* and *TaGDSL* were present in several accessions where *Qfhb1* has never been detected. They also identified the *TaPFT* gene in Durum wheat, which is highly susceptible to FHB. Additionally, they detected the *TaPFT* and the *TaGDSL* in 500 Chinese landraces collected nationwide, but the deletion mutation of *TaHRC* was only found in accessions from southern China (Su et al., 2017). Therefore, they conclude that the deletion mutation including start codon for *TaHRC* is the candidate for FHB resistance in *Qfhb1,* which they have reported to be a relatively new trait that might have evolved in parts of southern China and Japan. *Qfhb1* expression is a dominant trait in its phenotype (Schweiger et al., 2016; Xie et al., 2007). Therefore, it is hard to explain the association of *TaHRC* with *Qfhb1* in FHB resistance because we cannot explain the dominant phenotype of *Qfhb1* with a deletion mutation locus for its function; however, it's reported that *TaHRC* contributed resistance is semi-dominant in nature. Besides, both Rawat et al. (2016) and Schweiger et al. (2016) observed that this His-rich Ca-binding protein gene expressed indifferently between the FHB-resistant and the FHB-susceptible wheat lines.

### 1.5 Control of FHB by Fungicides

Fungicide application is useful during epidemic years of FHB to reduce the economic impact of the loss because of wheat cultivars having only modest levels of resistance against the disease. Many commercial brands of fungicides are available for application in FHB, such as propiconazole (Tilt 3.6E, Syngenta Crop Protection), tebuconazole (Folicur 3.6F, Bayer CropScience), Proline 480 SC (prothioconazole, Bayer

CropScience), Folicur (tebuconazole), Prosaro 421 SC (prothioconazole plus tebuconazole, Bayer CropScience) and Caramba 0.75 SL (metconazole, BASF Corporation) (McMullen et al., 2012). However, their use is limited due to various factors, such as poor efficacy of a fungicide, incorrect timing of application or inefficient application of a fungicide and residues of certain fungicides in harvested grains (Mesterhazy, 2003).

### 1.6 Biological control of *F. graminearum*

Management of FHB using other biological agents is another approach that has potential but not used in a large scale. The strategy behind biological control of FHB is the use of antagonistic microorganisms that can compete and slow the growth and or pathogenicity of *F. graminearum* (Wegulo et al., 2015). Bacteria and fungi are among the biological control agents identified and tested in vitro, in greenhouse and in field trials. Strains of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Lysobacter enzymogens*, and *Streptomyces spp.* among bacteria, and *Cryptococcus spp*., *Trichoderma spp.*, *Clonostachys rosea*, and *Aureobasidium pullulans* among fungi are some biological control agents against *Fusarium spp* causing FHB (Matarese et al., 2012; Palazzini et al., 2007; Schisler et al., 2002; Schisler et al., 2006; Schisler et al., 2011; Wachowska and Glowacka, 2014; Wegulo et al., 2015; Xue et al., 2014; Zhao et al., 2014). These control agents can be applied directly to spikes like fungicidal control agents; however, the challenge remains about their application timing and other environmental factors for their effectiveness.

Recently, exploration on potential of mycoviruses that cause hypovirulence in many fungal pathogens has gained attention. Mycoviruses are ubiquitous in nature, yet

very few mycoviruses that exist are discovered (Cook et al., 2013; Marzano et al., 2016; Rosario and Breitbart, 2011). Fungal viruses are often associated with symptomless infections of their host; however, many mycoviruses are identified that reduce the virulence of fungal pathogens. Cryphonectria hypovirus 1 (CHV1), which infects and reduces the virulence of *Cryphonectria parasitica*, is one of the very few viruses, which has been utilized as a biological control agent against the fungus pathogen (Ghabrial et al., 2015). Fusarium graminearum virus 1 (FgV1), which infects and reduces virulence of *F. graminearum*, was isolated and characterized over a decade ago (Chu et al., 2002), has not yet been successfully developed as an effective biological control agent. However, FgV1 has potential to be a biocontrol agent because of its ability to make *F. graminearum* significantly hypovirulent (Yu et al., 2009).

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## Chapter 2

*WFhb1-1* plays an important role in resistance against Fusarium head blight in wheat[†]

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### 2.1 Introduction

Fusarium head blight (FHB, also called scab or head scab) is a severe fungal disease of small grains such as bread wheat (*Triticum aestivum* L.), durum wheat (*T. durum* L.), oat (*Avena sativa*), and barley (*Hordeum vulgare* L.). FHB can be caused by several *Fusarium* species with *F. graminearum* as the primary pathogen in warm and humid regions worldwide including USA. Economic losses caused by FHB in wheat alone have been over billions of US dollars since 1990 (Windels, 2000; Wilson et al., 2018). Reduced yields, shriveled grains, mycotoxin contamination, and reduction in seed quality are major factors that are related to the losses due to this disease (Desjardins and Hohn, 1997; McMullen et al., 1997; Snijders, 1990; Dahl and Wilson, 2018). The mycotoxins produced by the pathogen will remain in processed foods causing health hazards in humans and animals (Pestka, 2010). Deoxynivalenol (DON) is the primary mycotoxin produced by *F. graminearum* in infected grains (Desjardins and Hohn, 1997).

Utilization of host resistance to develop resistant cultivars is the most promising approach to control FHB. Two major types of FHB resistance are widely accepted: resistance to the initial infection (Type I), and resistance to the spread of infection within the head (Type II) (Schroeder and Christensen, 1963). Type I resistance is common in barley but rare in wheat, which is most likely contributed by spike morphology

(Mesterhazy, 1995) and by activation of systemic innate immune responses (Foroud et al., 2012). In contrast, Type II resistance is attributed by different resistant genes and has been more extensively studied and utilized. FHB resistance in wheat is a quantitative trait. Numerous genetic studies on various resistance sources have shown that Type II resistance in each resistant wheat cultivar is most likely controlled by two to three major genes and a few minor genes (Gao et al., 2005; Ginkel et al., 1996). Molecular mapping of quantitative trait loci (QTLs) for Type II resistance has been extensively reported. Overall, about 100 QTLs associated with FHB resistance are mapped in all wheat chromosomes but 7D (Buerstmayr et al., 2009). Effectiveness of these QTLs is strongly influenced by genetic background and environments. Efforts to identify candidate genes of some key QTLs have also been made (Liu et al., 2008; Zhuang et al., 2013; Schweiger et al., 2016; Rawat et al., 2016; Giancaspro et al., 2018; Su et al., 2019; Li et al., 2019; Gadaleta et al., 2019), which has led to a better understanding of the pathogenesis and resistance mechanisms.

The FHB-resistance QTL *Qfhb1* (formerly known as *Qfhs.ndsu-3BS* and sometimes simply called *Fhb1*) on chromosome arm 3BS was first identified from a Chinese cultivar Sumai 3 (Anderson et al., 2001). Since then, it has been well defined as the most effective and the most stable QTL across different genetic backgrounds and various environments (Anderson et al., 2001; Basnet et al., 2012; Buerstmayr et al., 2002; Somers et al., 2003; Zhou et al., 2002). *Qfhb1* usually account for 20-60% of the phenotypic variation in FHB resistance (Buerstmayr et al., 2009). Therefore, *Qfhb1* has been the main resistance QTL deployed in wheat breeding to improve FHB resistance worldwide and the research focus for the resistance mechanism in wheat.

Fine mapping efforts have narrowed *Qfhb1* down to a 261-kb region of wheat chromosome arm 3BS (Bernardo et al., 2012; Hao et al., 2012; Liu et al., 2008; Zhou et al., 2010). Seven potential genes in this QTL region have been recognized in wheat cultivar Chinese Spring; they were cloned and evaluated, but none of them was found to be an FHB resistance gene (Liu et al. 2008). Later, 28 genes were recognized in a Sumai 3-derived, *Qfhb1*-containing 860-kb region, from which only a *GDSL* lipase gene showed a pathogen-dependent expression pattern and thus is qualified as a functional gene candidate for *Qfhb1* while a possibility of more than one gene causing the phenotypic difference was also suggested (Schweiger et al., 2016). However, this *GDSL* was not among the 13 genes identified in the QTL interval of Sumai 3 by Pumphrey (2007) and Rawat et al. (2016). Rawat et al. (2016) instead claimed that a pore forming toxin like protein gene (*PFT*) is a functional gene of *Qfhb1*. Nevertheless, *Qfhb1* can detoxify DON (Hofstad et al., 2016; Lemmens et al., 2005), but *PFT* cannot (Rawat et al., 2016). The unique existence of *GDSL* and *PFT* in *Qfhb1*-carrier wheat genotypes was a key reason for their identification as the genic component of *Qfhb1*. Su et al. (2019) and Li et al. (2019) did an extensive study by also surveying hundreds of wheat accessions from a worldwide collection and found that neither *PFT* nor *GDSL* is unique to *Qfhb1*-carrier wheat genotypes. These latest studies called into question the idea of *PFT* being an *Fhb1* candidate gene. Additionally, He et al. (2018) and Jia et al. (2018) also reported that *PFT* exists and functions in some susceptible wheat accessions they surveyed. In the most recent publications, Su et al. (2019) and Li et al. (2019) independently concluded that the mutation of a histidine-rich calcium-binding protein gene [named as *TaHRC* in Su et al. (2019) and *His* in Li et al. (2019)] confers resistance against FHB. *TaHRC* is a

susceptible gene, and a large deletion in the start codon region of its susceptible allele makes it silent, resulting in FHB resistance (Sue et al., 2016; 2018; 2019). However, Li et al. (2019) claim that the deletion in *TaHRC* makes frameshift mutation expressing a new protein that confers resistance. Both Su et al. (2019) and Li et al. (2019) reported that the FHB resistance conferred by the deletions is genetically semi-dominant. Previously, we reported that a wheat gene with an unknown function, named as *Wheat Fhb1 candidate 1* (*WFhb1_c1* or *WFhb1-1*), could be a functional genic component of this QTL (Basnet et al., 2012; Li and Yen, 2008; Zhuang et al., 2013). Therefore, the debate on *Qfhb1*'s genic component is going on.

Various functional mechanisms of *Qfhb1* have also been proposed, but none has been validated without argument. These proposed functions include detoxifying DON (Hofstad et al., 2016; Lemmens et al., 2005), thickening secondary cell wall in rachises after pathogen infection to prevent the pathogen to spread (Gunnaiah et al., 2012), inhibiting pectin methyl esterase to prevent the pathogen from penetrating the host cell wall (Zhuang et al. 2013), mediating jasmonic acid (JA) and ethylene (ET) signaling pathways to elicit local and systemic resistance (Gottwald et al., 2012; Li and Yen, 2008; Schweiger et al., 2016; Xiao et al., 2013), killing the infecting pathogen (Paudel et al., 2017; Rawat et al., 2016) or simply reducing FHB susceptibility that leads to FHB development (Eldakak et al., 2018; Su et al., 2018). Possible simultaneous regulation of at least two different resistance mechanisms by multiple functional components of *Qfhb1* was also suggested (Eldakak et al., 2018; Gottwald et al., 2012; Hao et al., 2012; Schweiger et al., 2016). Nevertheless, *Qfhb1* has been well recognized to simultaneously reduce FHB severity in the spikes and DON content in the kernels.

As mentioned above, our previous studies using a combination of approaches including transcriptomics and physiological studies (Li and Yen, 2008), and QTL, eQTL, and physical mappings (Basnet et al., 2012; Zhuang et al., 2013) revealed wheat gene *WFhb1-1* as a candidate for the functional genic component of *Qfhb1* in Sumai 3. Analyzing the expression pattern of this gene revealed its differential expression between FHB-resistant and FHB-susceptible wheat lines in response to the *F. graminearum* infection during the early stage of the pathogenesis. This result implies that the pathogen can suppress the expression of this wheat gene to initiate FHB pathogenesis in FHB-susceptible wheat genotypes, while such suppression mechanism does not work in FHB-resistant wheat genotypes. Therefore, cloning of this gene, and elucidating its function are necessary to further our understanding about its role in FHB pathogenesis/resistance and about how it is regulated by the pathogen infection. Here we report the results of our efforts to clone this gene from Sumai 3, to elucidate its biological function and to functionally validate its role in FHB resistance using Sumai 3 and a pair of Sumai 3-derived *Qfhb1* near-isogenic lines 206-1-1-2 (carrying *Qfhb1*, called NIL-R hereafter) and 260-1-1-4 (not carrying *Qfhb1*, called NIL-S hereafter).

## 2.2 Materials and Methods

The formulas of the buffers and media used in this study are listed in Supplementary Document S2.1. All the sequences of the adaptors and PCR primers used in this study are listed in Table 2.1.

### 2.2.1 Plant materials

Bread wheat cultivar Sumai 3 (FHB-resistant), landrace Y1193-06 (FHB-susceptible),

a pair of *Qfhb1* NILs 206-1-1- 2 (NIL-R) and 260-1-1-4 (NIL-S), Bobwhite (FHB-susceptible) and its CRISPR/Cas9-edited *TaHRC*-knockout mutant were used in this study. The NILs were developed and kindly provided by Dr. James Anderson's lab at University of Minnesota. The *TaHRC* knockout Bobwhite mutant was kindly provided by Dr. Guihua Bai of USDA-ARS/Kansas State University. For each experiment, at least 10 plants of each line per repeat per treatment were grown in pots filled with Miracle-Growth Potting Mix in a greenhouse or a growth chamber under a 16/8 h light/dark period, and 25/16°C day/night temperature, supplied with cool, white fluorescent lamps.

### 2.2.2 Rapid Amplification of cDNA ends (RACE) and Gene Cloning

RACE experiments were conducted using total RNA isolated from Sumai 3 and the 5'/3' RACE Kit, 2$^{nd}$ Generation (Roche Life Science). Gene-specific primers were designed according to the EST sequence (GenBank #: CA640991) that probe TaAffx.111425.2.S1_at on the Affymetrix Wheat Genome GeneChip was based on. The 5' and 3' RACE products were cloned and sequenced separately. The full-length cDNA was assembled by merging the 5'- and the 3'-amplicons from the RACEs, and then confirmed by PCR cloning of the whole sequence from Sumai 3. The cloned sequence was also compared with the publicly available chromosome arm 3BS pseudomolecule reference sequence of wheat cultivar Chinese Spring (now part of wheat reference genomic sequence IWGSC RefSeq v1.0.).

Genomic sequences of the gene of interest were PCR-cloned from Sumai 3 genomic DNA with primers designed based on the 3BS pseudomolecule sequence. Again, the PCR products of interest were cloned, sequenced, and validated by comparing them to the relevant sequences in the 3BS pseudomolecule. Putative promoter sequences were

predicted using TSSP and RegSite PlantProm DB (http://www.softberry.com/berry.phtml?topic=promoter) and Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Open reading frames were predicted by ORFfinder at NCBI.

### 2.2.3 Prediction of protein property

Protein properties were predicted with Phobius, a combined transmembrane topology and signal peptide predictor at http://phobius.sbc.su.se using the normal prediction function (Käll et al., 2004). Analysis of conserved protein domains was done using quick scan mode of ScanProsite (http://prosite.expasy.org/scanprosite/).

### 2.2.4 Protein expression in *Pichia pastoris* and *in vitro* inhibition assay of *F. graminearum*

#### 2.2.4.1 Antibody design

Primary antibodies PA-1 and PA-2 were raised in rabbit against the peptides P Q R P P A V G P F P W E and Q Q P P A S P R S G S G F P, respectively, which were selected from the putative protein sequence of WFhb1-1 (Figure 2.1A) following predictive analysis of protein folding. The antibodies were produced at GenScript USA Inc.

#### 2.2.4.2 Construction of expression vector

*WFhb1-1*'s ORF was synthesized according to the cloned gene sequence with an *EcoR* I site added at the 5' end and a *Xho* I site added at the 3' end. The synthesized foreign gene expression insert was put into *pUC57* and cloned into *Escherichia coli* JM109 by heat shock method. Briefly, 1 µg *pUC57_insert* plasmid was added to 50 µl *E.*

*coli* cells. The mixture was incubated on ice for 20 min, heat-shocked at 42°C for 50 s and immediately kept on ice for 2 min. Then, 950 µl SOC medium was added into mixture followed by ~1.5 h incubation at 37°C with shaking at ~150 rpm. The culture (50 µl and 100 µl) was spread on LB agar plates (with ampicillin 100 µg per ml) in duplicate. The plates were incubated at 37°C overnight and plasmid DNA was extracted from developed colonies for further use.

EasySelect™ *Pichia* Expression Kit (ThermoFisher Cat # K174001) was used to express the protein of interest in *P. pastoris* yeast. First, *pPICZA* was used to make the yeast expression vector. Briefly, the *pUC57-foreign gene* plasmid was digested with *EcoR* I and *Xho* I enzymes to release the expression insert, which was then ligated between the pre-cut *EcoR* I and *Xho* I sites in *pPICZA* using the T4 DNA ligase in 2X rapid ligation buffer from Promega. After the desired orientation of the insert in *pPICZA-foreign gene* was confirmed, nearly 10 µg of *pPICZA- WFhb1-1* was linearized with *Sac* I. The linearization was confirmed by running the digested and undigested plasmid parallelly in 1% agarose gel. For preparing competent cells of *P. pastoris* strain X33, the yeast cells were cultured in 5 mL YPD (yeast extract peptone dextrose) medium in a 50-mL conical flask at 29°C overnight. Fifty milliliters of fresh YPD medium in a 250-mL conical flask was inoculated with 0.5 mL of the overnight culture and was again incubated at 29°C overnight. Next day, the culture was centrifuged at 1500 ×g for 5 min at 4°C to harvest the cells. The supernatant was discarded, and the cells were resuspended in 50 mL of ice-cold, sterile water. The cells were again centrifuged as above and resuspended in 25 mL of ice-cold, sterile water. The cells were centrifuged again with the same speed and dissolved in 2 mL of ice-cold, sterile 1 M sorbitol. The cells were

centrifuged one more time as above and dissolved in 0.5 mL of ice-cold, sterile 1 M sorbitol. Now the *P. pastoris* cells are ready for transformation by electroporation.

For transformation by electroporation, about 10 µg of the *Sac* I-linearized *pPICZA* expression vector was mixed with 80 µL of the *P. pastoris* competent cells and the mixture was transferred into an ice-cold 2-mm electroporation cuvette. The cuvette with the mixture was incubated on ice for 5 minutes. The cuvette was then put in BioRad GenePulsar X cell and pulsed once with pre-set protocol for *P. pastoris* with the parameters as follows: 2000 V, 25 µF, 200 Ω and 2 mm. Immediately after the pulsing, 1 mL of ice-cold, sterile 1M sorbitol was added in the cuvette, and the cuvette content was transferred into a sterile 15 ml tube. The tube was incubated at 29°C without shaking for 1.5 hours. Then, the incubated culture in the tube was spread on YPD agar plates containing 100 µg/mL zeocin. Different volumes (10, 20, 50, 100, and 200 µL) of the culture were spread on separate YPD plates with zeocin. The plates were then incubated at 29°C until the colonies formed (about a week). Then, 10 colonies were picked and the X33:T expression strain was further confirmed by its Mut+ phenotype following the protocol (https://tools.thermofisher.com/content/sfs/manuals/easyselect_man.pdf) from Invitrogen. X33:T was also confirmed by PCR using *WFhb1-1*-ORF forward & reverse, 5'AOX and 3'AOX primers. X33 was also transformed with *pPICZA-wt* (the wildtype *pPICZA* plasmid without the expression insert) to form the background control strain X33:00, following the same protocol as explained above.

### 2.2.4.3 Protein expression in the *P. pastoris* expression system

For the protein expression experiments, a single colony of X33:T or X33:00 was cultured in 25 mL of MGY (minimal glycerol yeast) medium in a 250-mL conical flask

and incubated at 29°C for 16-18 h with shaking (250 rpm). To induce foreign protein expression in *P. pastoris*, cells were harvested by centrifuging at 3000 ×g for 5 min at RT and the supernatant was discarded. The cell pellet was then resuspended in 25 mL of MM

**Table 2.1.** Sequences of adaptors and PCR primers used in this chapter.

| Name | Sequence |
|---|---|
| *WFhb1-1* insert Forward | TGTGCTCTGCTTTCCTGCTG |
| *WFhb1-1* insert Reverse | CCCAGCATACAGTTGAAACG |
| *WFhb1-1* expression Forward | CTGAGCGGCTGCTGTGCTGA |
| *WFhb1-1* expression Reverse | ATATCAGATCTGGCAGTGCCCCA |
| BSMV:T stability Forward | TGATGATTCTTCTTCCGTTGC |
| BSMV:T stability Reverse | GTTTCCAATTCAGGCATCGT |
| pTMH Forward | TGCTACATCCATACTCCATCCTTCCC |
| pTMH Reverse | AGCTGACATCGACACCAACGATCT |
| UBC Forward | TCCCCTTACTCTGGCGGTGTC |
| UBC Reverse | TTGGGGTGGTAGATGCGTGTAGT |
| *WFhb1-1*-ORF-Forward | CACGCAGTTCCCCTTCTG |
| *WFhb1-1*-ORF-Reverse | GAGCAGCAGCAAAGCAGAG |
| 5'AOX | GACTGGTTCCAATTGACAAGC |
| 3'AOX | GCAAATGGCATTCTGACATCC |
| Gamma-1-Forward | CGCAATACGTAAGTCCGTAGC |
| Gamma-1-Reverse | GATGGGCACCATCAGATTT |
| Wheat β-actin Forward | AAATCTGGCATCACACTTTCTAC |
| Wheat β-actin Reverse | GTCTCAAACATAATCTGGGTCATC |
| *HRC-qPCR* Forward | CAGCAGAGTTCACACGATGA |
| *HRC-qPCR* Reverse | GGTGAGCCAGACAAGATGAA |
| *His* Forward | CAAGTACAGGCTTCAGAATCCA |
| *His* Reverse | GCAACTCGTGTAAGTTGTTAAAA |
| *PFT* Forward | GGATCTGGGCTGATTCAACT |
| *PFT* Reverse | TTTCGCAGAGCAATGAAGTC |
| *GDSL* Forward | TCAACAGGAGCCAGTTTGTC |
| *GDSL* Reverse | GATGTCCAAGGTGTAAAGCG |

(Minimal Methanol) medium in a 250-mL conical flask, covered with two-layer sterile cheesecloth, and continued to incubate at 29°C in the shaker incubator (250 rpm). Methanol was added to a final concentration of 0.5% every 24 hours to maintain the expression induction. At each time points (Day0, Day1, Day2, Day3, and Day4), 1 mL of the expression culture was collected into a 1.5 mL microcentrifuge tube. This sample is used to analyze the growth kinetics by counting the cells and to extract the total protein.

#### 2.2.4.4 *Pichia pastoris* growth kinetics

*P. pastoris* growth kinetics were studied by counting the number of cells per milliliter in the expression culture in Day0, Day1, Day2, Day3 and Day4 starting from the time when methanol was first added into the MM medium (Day0). This was done by first diluting 10 µL of the culture from each time point with 990 µL of sterile water to make a 100-fold dilution. The diluted culture samples were then microscopically observed on a hemocytometer to count the cells in the four squares with 0.1 $mm^3$ per square. The cell numbers in the four squares were averaged and multiplied with the dilution factor to get the final count as "cells /mL". The budding cells attached together were counted as a single cell.

#### 2.2.4.5 Protein extraction and quantification

Total proteins were extracted from both X33:T and X33:00. One milliliter of the expression culture sample was collected into a 1.5 mL microcentrifuge tube at each time point and centrifuged at maximum speed in a tabletop centrifuge for 2 minutes at room temperature. The supernatant was transferred into a new 1.5 mL tube. Both the supernatant and the pellet were snap-frozen in liquid nitrogen and stored at -80°C until further processed. Total protein concentration of this supernatant was measured at 562 nm absorbance using Pierce BCA Protein Assay Kit from Thermo-Scientific, and final concentration of 1 µg/µL was maintained for all the samples by adding adequate MM medium.

For the extraction of total protein from pellet sample, each pellet was resuspended in 100 µL breaking buffer. Equal volume of acid-washed glass beads (size 0.5 mm) was

then added into the pellet suspension and vortexed for 30 s. The mixture was incubated on ice for 30 s and then vortexed before another 30 s incubation. This cycle was repeated for eight times. After the final vortexing, the mixture was centrifuged at maximum speed for 10 min at 4°C. The clear supernatant was then transferred to a fresh 1.5 mL microcentrifuge tube. Total protein concentration was measured at 562 nm absorbance using Pierce BCA Protein Assay Kit from Thermo-Scientific, and 1 µg/µL final concentration was achieved by adding breaking buffer.

For exaction of total protein from wheat spikelets, each sample harvested at a desired time point were ground in 1.5 mL tubes with plastic pestle in liquid nitrogen until the fine powder was obtained. After grinding the sample, 200 µL of breaking buffer was added in each tube. Vortexing was done with intermittent incubation of tubes on ice (vortexing for 30 s followed by incubation on ice for 30 s for total 8 cycles). Then, the tubes were centrifuged at maximum speed at 4°C for 10 min, and the clear supernatant was transferred into new micro-centrifuge tubes. Protein concentration was measured with 280 nm absorbance, and the concentration was also confirmed using Pierce™ BCA Protein Assay Kit (ThermoFisher Cat # 23250). Additional breaking buffer, if needed, was added to make the final concentration of each protein sample 1 µg/µL. These extracted protein samples were stored at -80°C until further used.

#### 2.2.4.6 In vitro inhibition assay of *F. graminearum* with the yeast-produced protein

The total protein in breaking buffer in a 1.5-mL microcentrifuge tube was precipitated by adding four times (in volume) of cold acetone (stored at -20°C) into the tube. The tube was then vortexed and incubated for 1 h at -20°C. Then, the tube was centrifuged for 10 minutes at 15000 × g, and the supernatant was carefully discarded. The protein pellet was

air-dried by leaving the tube lid open at RT for about 30 min. The dried pellet was then dissolved in adequative amount of sterile distilled water to make the total protein solutions of desired concentrations, which was measured by the standard BSA protein assay. Series of total protein concentrations (20, 50, 100, 500, 600, 700 µg/mL) in each volume were made and used in the inhibition assay. To produce a larger amount of total protein, the scale was increased by 50 folds.

To assay if the yeast-produced protein can inhibit *F. graminearum* growth, about 10,000 *F. graminearum* conidia were grown in 100 µL of potato dextrose broth (PDB) per well in a 48-well cell culture plate. Total protein extracted from either X33:T or X33:00 cultures in a concentration of 20, 50, 100, 500, 600 or 700 µg/mL was added into three parallel wells, and sterile water was used as blank control. Each treatment was repeated three times. Growth of *F. graminearum* in each well was visually assessed in two weeks after the treatment started, and high-resolution pictures for plates were taken. The intensity of the fungal growth in each well was calculated and analyzed by using ImageJ software (Schneider et al., 2012). In brief, equal area of each wells was selected and the intensity of the selected area was calculated in ImageJ for each well with fungal growth and the statistical analysis was done.

### 2.2.5 Polyacrylamide gel electrophoresis and Western blotting

For PAGE, 25 µg of total protein was loaded in each well of BioRad pre-casted mini polyacrylamide gels. Precision plus protein standard (BioRad) was used to estimate the protein mass. The gel was run for about 1 h with 30 mA constant current in a Bio-Rad mini PROTEAN tetra cell, and then was either processed for Sypro-Ruby staining or for the Western blotting.

For Sypro-Ruby staining, the gel was first fixed in fixing solution (50% methanol and 10% acetic acid in distilled water) for 1 h. Then, the fixing solution was discarded, and the gel was soaked in 50 mL Sypro-Ruby protein gel stain solution for 2 h with gentle agitation and protection from light. Then the gel was transferred into a clean tray and washed with wash solution (10% methanol and 7% acetic acid in distilled water) with gentle shaking for 15 min. The gel is then visualized and recorded under UV.

For Western blotting, after the PAGE was run, the total protein on the gel was transferred onto a nitrocellulose membrane using iBlot from Invitrogen. Then, the membrane was incubated in buffer TBST with 5% dry milk for 1 h with gentle shaking for blocking. The membrane was then washed three times with buffer TBST having 0.5% dry milk (10 minutes each time with gentle shaking). Then the membrane was incubated in primary antibody solution diluted to 1:10,000 in TBST with 0.5% dry milk for 1 h at RT with gentle shaking. Then the membrane was washed for 3 times with TBST having 0.5% milk as described above. LICOR IRdye 800 CW goat anti-rabbit antibodies were used for secondary binding and visualization. Same dilution as primary antibodies (1:10,000) was used in TBST with 0.5% dry milk, and the membrane was incubated at RT for 1 h with gentle shaking. The membrane was then washed again 3 times as described above and visualized in LICOR Odyssey Fc under 800 nm absorbance with exposure time of 2 milliseconds.

Western Blots of total wheat protein were digitized for fluorescence signal strength with LICOR Odyssey Fc and analyzed. At least three bio-repeats per treatment and three technical repeats per bio-repeat were performed. For each NIL, the signal data were compared between the treatment and the mock control per each time point and the

relative change were then normalized by taking the value at 0 hpfi (hours post *Fusarium* inoculation) off (Supplementary Table S2.1).

### 2.2.6 Constructing overexpression and knockdown vectors

A barley stripe mosaic virus (BSMV)-based virus-induced gene overexpression (VOX)/silencing (VIGS) system was used to transiently overexpress or silence the gene of interest. The BSMV vectors used were kindly provided by Dr. Li Huang of Montana State University. The BSMV γ vector (γ PCR vector) was modified by Huang's group with two *Xcm* I restriction sites inserted. We further modified it by adding an *EcoR* I site between the two *Xcm* I sites. To construct the overexpression vector BSMV:W, *WFhb1-1*'s ORF (384 bp) with a *EcoR* I site at each end was synthesized at GenScript. BSMV:W was then constructed by digesting the γ PCR vector and the synthesized ORF with *EcoR* I, and mixed then together to have the ORF inserted in the *EcoR* I site. The desired orientation of the ORF was confirmed with PCR using *WFhb1-1*-ORF forward/Gamma-1 reverse primers. To construct the silencing vector BSMV:T, a fragment of 289-bp of the *WFhb1-1* coding sequence in anti-sense orientation was amplified by PCR from Sumai 3 and ligated into the γ PCR vector. Both the VOX and VIGS vectors were confirmed by sequencing.

### 2.2.7 In-vitro transcription of viral RNA and plant inoculation

The three BSMV RNA chromosomes were reversely transcribed from the corresponding BSMV vectors following the protocol provided by Dr. Li Huang of Montana State University. RNA quality was assessed on 1% agarose gel. Virus inoculation was done by following the previously described inoculation procedures (Ma

et al., 2012; Scofield et al., 2005). Briefly, a mixture of the three viral RNAs in viral inoculation buffer FES was manually rubbed into plant tissue.

Viral inoculation was carried out with either the VOX vector BSMV:W, the VIGS vector BSMV:T, the empty BSMV control vector BSMV:00 or the viral inoculation buffer FES alone on at least 10 plants per line per treatment. The inoculation was done either on a spike at shooting stage as soon as three fourths of the spike was emerged, on a 10-day old leaf, or on a flag leaf at the booting stage when the flag leaf was fully expanded.

### 2.2.8 Fungal inoculum preparation, inoculation, sampling, and disease evaluation

*F. graminearum* isolate Fg4, collected from Watertown, SD was used in this study to induce FHB. *F. graminearum* was cultured on potato-dextrose-agar (PDA) medium for a week, and then spores were collected for plant inoculation. Procedures used for wheat spike inoculation as previously described (Li and Yen, 2008). Briefly, *F. graminearum* spores were washed from PDA plates using sterile water and then filtered through four layers of sterile cheesecloth. The concentration of conidia was counted using a hemocytometer and adjusted with sterile water to about 100,000 conidia/mL. The spikelet was challenged with 10 µL of water-suspension of *F. graminearum* conidia or sterile water alone (as a mock control) at the stage when intensive yellow color of anthers was observed. For each treated spike, the two first-flowering spikelets were inoculated to introduce a disease pressure at the level that an FHB-resistant genotype will be maximumly diseased at 28 dpfi. The inoculated spikes were immediately covered with plastic zip-lock bags with a wet cotton ball inside for 72 h to maintain the optimal humidity and temperature to facilitate disease establishment. The length between the

BSMV and the *F. graminearum* inoculations was optimized for both leaf and spike inoculations by test inoculation of the two at various time intervals until the maximum effect of overexpression or knockdown of *WFhb1-1* was reached, usually at 4 ~7 dpvi when yellow pollens emerged out of the first pair of spikelets.

For disease evaluation, FDR was calculated as percentage of diseased rachides of all rachides per spike and FDK was calculated as percentage of diseased kernels of all harvested kernels per spike. FDR and FDK were averaged per time-point per treatment per experiment at 7, 14/15, 21 and 28 dpfi, respectively. DON content in the harvested kernels per spike was also measured by sending the harvested kernels to the DON testing lab at University of Minnesota and analyzed for each treatment in our lab.

**2.2.9 Quantitative Real-time PCR (RT-qPCR)**

Total RNA was extracted from leaf, spike, or spikelet samples with TRIZOL (Invitrogen) following the manufacturer's instructions. RNA quality was tested using 0.8% or 1% agarose gel and quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Wilmington, DE). For each treatment, three to four biological replications were conducted. For reverse transcription, ~500 ng DNase I-treated total RNA was used for cDNA synthesis using GoScript™ Reverse Transcriptase system (Promega) with oligo$(dT)_{15}$ primer. For VOX and VIGS experiments, if relevant, the presence of BSMV viral genome in the cDNA samples was confirmed before RT-qPCR was conducted. The RT-qPCR was conducted on a Smart Cycler II (Cepheid) or QuantStudio 6 Flex (Applied Biosystems). Briefly, 2X dilutions were made for reverse transcription products, and 1 µL diluted cDNA/20 µl reaction was carried out using SYBR green I master Mix with 2 min at 95°C, 45 cycles of 20 s at 95°C, 30 s at melting

temperature, and 30 s at 72°C, and then 5 min at 72°C. Wheat β-actin gene was used as an internal control to normalize the *Ct* value. For each sample, three technical replications were conducted. Fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 2.2.10 Statistical analyses

Tukey's multiple comparisons of means in *R* software package was used to compare FDR and FDK values of each treatment at the 95% family-wise confidence level. We also conducted student *t*-test and one-way ANOVA analysis for these data. The association between transcript abundance of a gene of interest and FDR in the gene silencing experiment was measured using Pearson's product-moment correlation analysis in *R* software package.

## 2.3 RESULTS

### 2.3.1 Cloning the full-length cDNA and the genomic sequence of the gene

The first step of this study was to clone the full-length cDNA of the candidate gene for a functional genic component of *Qfhb1*, which we identified in our previous study (Zhuang et al., 2013). The 5' and the 3' ends of the cDNA were first cloned from the spike sample of Sumai 3 with 5'/3' RACE technologies, respectively, and the full-length cDNA sequence was then formed by merging the two partial cDNA fragments together. The full-length cDNA was then confirmed by cloning the full sequence from total RNA of Sumai 3 spikes by RT-qPCR and sequencing. The full-length cDNA sequence (Figure 2.1A) has been deposited in GenBank (GenBank # KU304333.1).

The corresponding genomic sequence was then cloned from Sumai 3 by PCR with

gene-specific primers designed on the basis of the cDNA sequences and the corresponding up-stream and down-stream genomic sequences of chromosome arm 3BS pseudomolecule (GenBank #: HG670306.1) of wheat cultivar Chinese Spring (now part of wheat reference genomic sequence IWGSC RefSeq v1.0.). Comparing the full-length cDNA and the genomic coding sequence revealed only one exon in this gene (Figure. 2.1A). Analyzing the upstream genomic sequence revealed two potential promoters (Figure 2.1A). Our *in-silico* mapping has located the cloned gene between the 5641624 bp and the 5642007 bp in the 3BS pseudomolecule flanked by markers *Xsts9-2* and *Xsts65-3* (Figure 2.1B).

The predicted protein of this cloned gene (GenBank # ANE31719.1) has 127 amino acid residues, which shows 98% identity (*E* = 1e-80) to an unnamed wheat protein (GenBank # CDM801516.1) reported by Choulet et al. (2014). Analyzing the predicted protein with Phobius predicted that it is probably a transmembrane protein with an undefined extracellular signaling domain (Supplementary Figure S2.1). However, analyzing it with ScanProsite did not give a clear clue on its biological function since no obvious conservative protein domain was found in the predicted protein. Therefore, we need to reveal the biological function of this protein experimentally.

### 2.3.2 Protein expression in *Pichia pastoris*

To study the biological function of the cloned gene, its open reading frame (ORF) was cloned into and expressed in *P. pastoris* (Supplementary Figure S2.2). Two antibodies PA-1 and PA-2 were designed and produced for specific detection of the putative protein encoded by the cloned gene (Figure 2.1A). The yeast-produced WFhb1-1 was detected in the total protein extracted from the expressing yeast strain X33:T by

Western blotting using PA-1 as the primary antibody (Figure 2A). The yeast-produced WFhb1-1 stays intracellularly as it was detected in pellet samples but not in the supernatant of the cell extraction (Figure 2.2D). The mass of the protein was estimated to be ~60 kDa (Figures 2.2A, 2.2C & 2.2D), which is about 4 times larger than the ~13 kDa estimated from the predicted amino acid sequence. This observation indicate that this wheat protein probably forms a tetramer in the yeast total protein extraction.

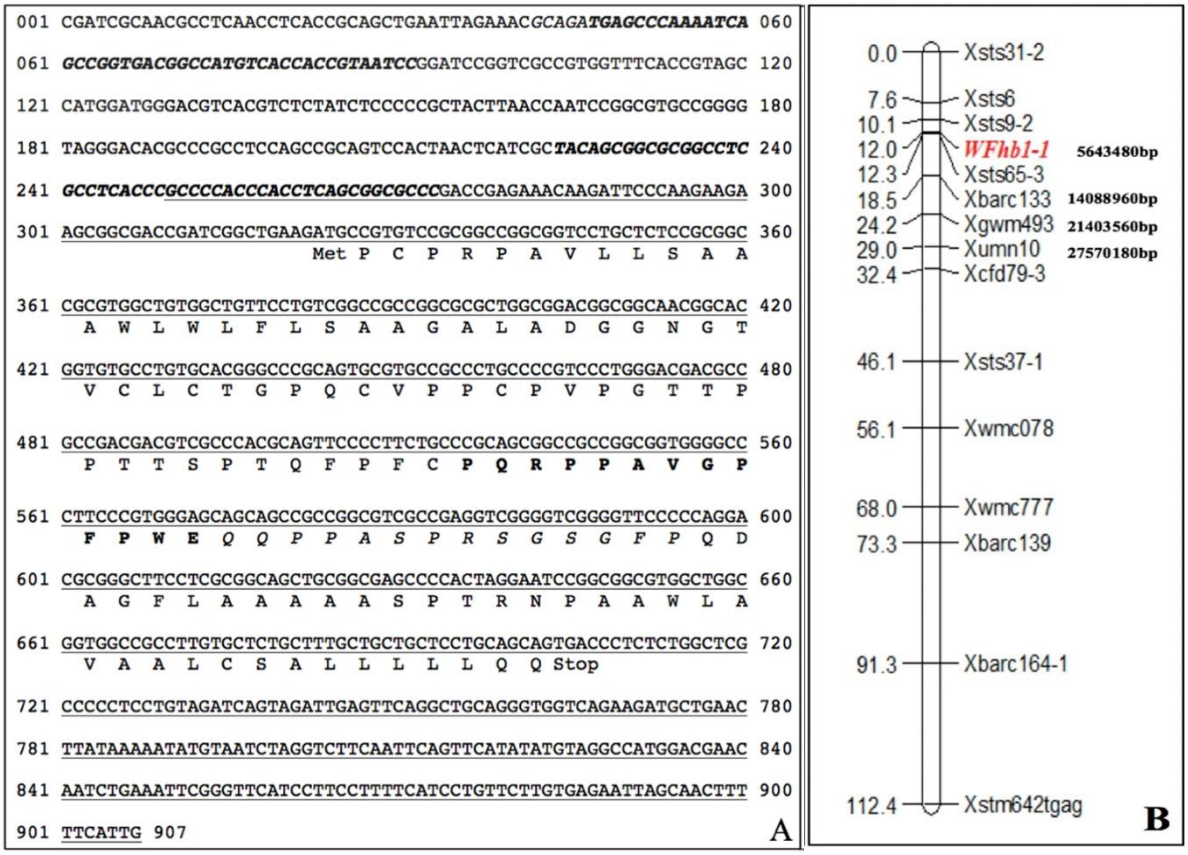


**Figure 2.1.** Sequences and map position of *WFhb1-1*. **A**: Genomic sequence of *WFhb1-1* with the two predicted promoter regions in bold italic. The full-length cDNA is underlined, and the predicted protein sequence is underneath. The amino acid sequences used for designing antibody PA-1 and PA-2 are in bold and italic, respectively. **B:** *In silico* mapping of *WFhb1-1* to Chinese Spring chromosome 3B pseudomolecule with position in the sequence indicated.

We have noticed that the yeast production of the WFhb1-1 could be detected only up to Day 2 of the induced expression (Figure 2.2D), and growth of X33:T (indicated by its increased cell number per cube unit of the culture) also occurred only during the first two days, while the control strain X33:00 continued to grow (Figure 2.2E). These results suggest that the yeast-produced wheat protein may be toxic to *P. pastoris* itself so that it stops growing and ceases the production when the wheat protein concentration reaches the life-threatening threshold in two days.

### 2.3.3 In-vitro inhibition assay of *F. graminearum* growth with the yeast-produced WFhb1-1

Since the yeast-produced WFhb1-1 showed a potential anti-yeast activity in the expression system (Figure 2.2E), we conducted experiments to see if it could also inhibit *F. graminearum* from growing in culture. Our data indicate that growth of *F. graminearum* in potato dextrose broth was indeed inhibited when 500 µg/mL or more of the total protein extracted from X33:T was added into the culture (Figures 2.2F and 2.2G, Supplementary Figure S2.3). The observed growth inhibition of *F. graminearum* by the total protein extracted from X33:00 yeast strain compared to the sterile water could be caused by residual precipitation reagents in the total protein sample.

Nevertheless, the obvious difference in inhibiting *F. graminearum* growth by the X33:T total protein compared to that by the X33:00 total protein indicates that the yeast-produced WFhb1-1 seems to have a broad antifungal ability, which could reduce FHB development in wheat. Further research in this aspect with purified WFhb1-1 is needed to further explore its anti-fungal mechanism and its potential utility as a bio-fungicide for controlling fungal diseases.

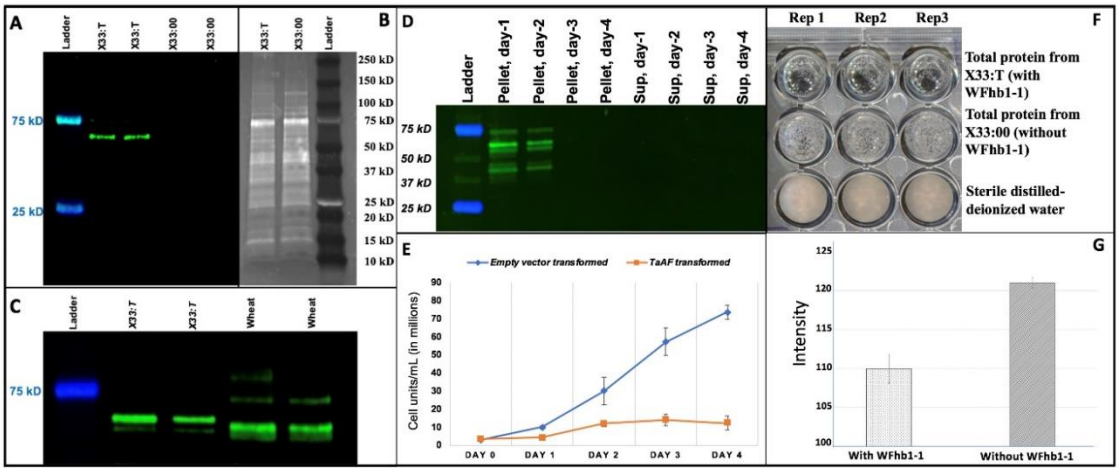


**Figure 2.2.** Photos and graphics showing the results of elucidating the WFhb1-1 produced by *WFhb1-1*-expression *Pichia pastoris* strain X33:T. **A**: a photo of a Western Blot of total protein isolated from X33:T and the wild type *P. pastoris* X33:00 probed with anti- *WFhb1-1* antibody PA-2; **B**: a photo of a Sypro-Ruby stain-polyacrylamide gel of the total protein isolated from X33:T and X33:00; **C**: a photo of a Western Blot of total protein isolated from X33:T and wheat spikes probed by PA-1. **D**: a photo of Western Blot of total protein extracted from X33:T probed with anti-WFhb1-1 antibody PA-1 on day 1, day 2, day 3 and day4 after methanol was added into the culture to turn on *WFhb1-1* expression; Sup: supernatant. **E**: a graphic showing the growth kinetics of X33:T and X33:00 post addition of methanol into the culture medium to turn on *WFhb1-1* expression. **F**: a photo showing growth of *Fusarium graminearum* in 100 µL potato dextrose broth supplemented with 500 µg/mL total protein isolated from X33:T or X33:00 or sterile water. About 1000 conidia were used to initiate the culture in each well. The photo was taken two weeks after the culture started. **G:** Comparison of intensity of fungal growth between WFhb1-1 protein-added and control protein-added wells.

### 2.3.4 Evaluation of WFhb1-1 protein accumulation in wheat spikes

Western blotting assays using either anti- WFhb1-1 antibodies PA-1 (Figure 2.2C) or PA-2 (Figure 2.3) as primary antibody have detected WFhb1-1 accumulation in wheat

spikes, confirming *WFhb1-1* as a protein coding wheat gene. Our previous study showed that *WFhb1-1* is suppressed by the pathogen infection in FHB-susceptible wheat genotypes but not in the FHB-resistant genotypes (Zhuang et al., 2013). We would like to know how this transcription suppression impacts WFhb1-1 protein accumulation *in vivo*. Protein samples were collected from the *F. graminearum*-inoculated and the mock-inoculated spikelets of the NIL pair at 0, 8, 12, 24, 36 and 48 hpfi (hours post *Fusarium* infection), and subjected to Western blotting using anti- WFhb1-1 antibody PA-2 as the primary antibody (Figure 2.3; Supplementary Figure S2.4, Supplementary Table S2.1). We did not observe a significant difference at any time point between the FHB-inoculated and the mock inoculated samples of NIL-R (Figures 2.3B & 2.3D, Supplementary Table S2.1). However, significant difference ($p < 0.01$) was observed at 12 hpfi between the FHB-inoculated and the mock-inoculated NIL-S spikes (Figures 2.3C & 2.3D). These observations suggest that the pathogen infection reduces WFhb1-1 accumulation at the early stage of FHB pathogenesis but only in the FHB-susceptible wheat, similar as we previously observed at the transcription level (Zhuang et al., 2013). The estimated mass of the *in vivo*-produced WFhb1-1 is ~45 kDa (Figures 2.2C & 2.3A), which is about three times larger than the ~13 kDa calculated from WFhb1-1 polypeptide sequence but smaller than the ~60 kDa estimated for the yeast-produced WFhb1-1 (Figure 2.2C). The cause for the size difference between the yeast-produced, the wheat-produced and the calculated WFhb1-1 is unknown but could be due to formation of different multimers (i.e. trimer in wheat vs. tetramer in yeast). Binding with a protein of different size in the yeast vs. in wheat is an alternative explanation.

### 2.3.5 *WFhb1-1* gene overexpression in wheat

To understand the function of *WFhb1-1* in FHB resistance, we applied a barley stripe mosaic virus (BSMV)-based virally-induced gene overexpression system to NIL-S and NIL-R to see how overexpression of WFhb1-1 will impact FHB susceptibility/resistance in the NILs. To optimize this system in the NILs, the 2$^{nd}$ and/or 3$^{rd}$ leaf of 10-day old wheat seedlings were inoculated with either *WFhb1-1*-overexpressing BSMV strain BSMV:W or FES viral inoculation buffer. We first examined the existence of BSMV:W and the *WFhb1-1* expression by RT-qPCR in newly emerged leaves of the inoculated plants at 10 dpvi (days post viral inoculation). The BSMV was successfully detected in the BSMV:W-inoculated wheat plants but not in the FES-inoculated plants by RT-PCR, indicating that the infected BSMV:W was successfully assembled within the infected wheat leaves and were able to move to other parts of the plants. We detected up to ~30 fold increase *WFhb1-1* expression in BSMV:W-inoculated NIL-S plants compared to the FEF-inoculated NIL-S plants (Figure 2.4A). This observation confirms that BSMV:W infection can induce overexpression of *WFhb1-1* in the infected NIL-S plants. We also tested direct BSMV:W inoculation in spikes of NIL-S plants when three fourth of a spike emerges from the flag leaf. In this experiment, wheat plants inoculated with BSMV:00 (the wildtype BSMV strain) or FES were used as the controls. We observed significant induction of the *WFhb1-1* overexpression in the spikes of the BSMV:W-inoculated plants compared to the BSMV:00- or the FES-inoculated plants (Figure 2.4B). Results of this spike inoculation experiment suggest that significant overexpression could be detected at 14 dpvi.

To understand if *WFhb1-1* indeed plays a role in FHB resistance in wheat, we visually observed FHB severity (Figures 2.5A and 2.5C) on the inoculated spikes, and

investigated *Fusarium* damaged rachides (FDR) rate (Figures 2.5B and 2.5D, supplementary Figure S2.5), *Fusarium* damaged kernels (FDK) rate (Figures 2.6A and 2.6B) and DON content in harvested kernels (Figure 2.6C, and Supplementary Table S2.2) in the *WFhb1-1*-overexpressing wheat spikes under a high disease pressure (two spikelets were inoculated instead of usually one). For this purpose, NIL-S and NIL-R plants that were pre-inoculated with BSMV:W, BSMV:00 or FES were inoculated with *F. graminearum* conidia or water, respectively.

FDR data was collected from all the treated plants at 7, 14, 21, and 28 dpfi and analyzed (Supplementary Figure S2.5). One-way ANOVA and T-test both showed that the FDR was significantly lower ($p < 0.01$) in the BSMV:W-inoculated spikes compared to the BSMV:00- or the FES-inoculated spikes in all the four time points for NIL-S and three of the four time points for NIL-R; FDR at 7 dpfi for NIL-R was not statistically significant (Supplementary Figure S2.5). Similarly, FDK data was also collected for all the treated plants and it was also found significantly lower ($p < 0.01$) in the BSMV:W-inoculated spikes than in the BSMV:00- or the FES-inoculated spikes for both NILs (Figures 2.6A & 2.6B). For NIL-S, the DON level was found significantly lower in the BSMV:W-inoculated spikes compared to the BSMV:00- or the FES-inoculated spikes, while no difference was observed among the treatments of NIL-R (Figure 2.6C, Supplementary Table S2.2). It seems that overexpressing *WFhb1-1* in NIL-S reduces FHB to a level comparable to that observed in NIL-R (Figures 2.5A, 2.5B & 2.6; Supplementary Figure S2.5; Supplementary Table 2.2).

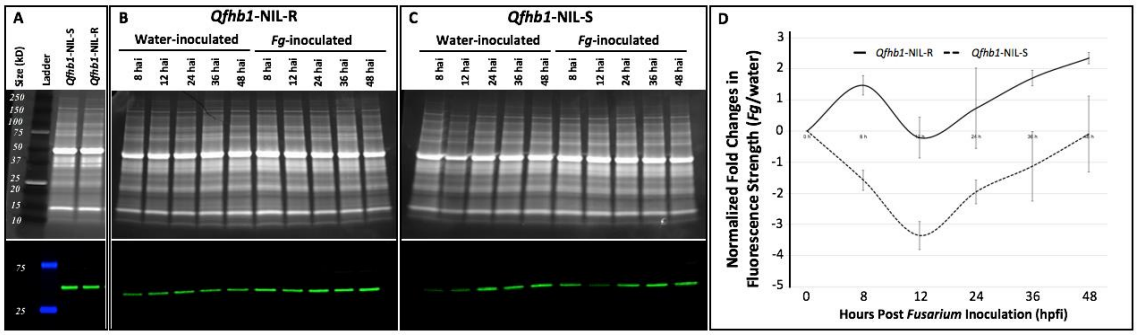


**Figure 2.3.** Photos and graphic showing the results of Western Blotting of total proteins isolated from wheat spikelets inoculated with *Fusarium graminearum* (*Fg*) or water at 0, 8, 12, 24, 36 and 48 hpfi (hours post *Fusarium* inoculation). In A, B and C, the upper panels are images of polyacrylamide gel stained with SYPRO Ruby, and the lower panels are photos of Western Blot probed with probed with anti- WFhb1-1 antibody PA-2. **A**: Total protein isolated from the water-inoculated spikelets of *Qfhb1*-NIL-R and *Qfhb1*-NIL-S at 48 hpfi; **B**: total protein isolated from the *Fg*- and the water-inoculated spikelets of *Qfhb1*-NIL-R; **C**: total protein isolated from the *Fg*- and the water-inoculated spikelets of *Qfhb1*-NIL-S; **D**: Normalized fold changes in fluorescence strength of WFhb1-1 revealed by Western blotting between the *Fg*-inoculated and the water-inoculated spikelets of each NIL at the six time points. The normalization was done by setting the value at 0 hpfi as the baseline for comparisons.

### 2.3.6 *WFhb1-1* gene silencing in wheat

To further confirm *WFhb1-1*'s role in FHB resistance in wheat, the BSMV-system was also applied to knock *WFhb1-1* down in spikes of Sumai 3 and Y1193-06 with RNA interference. The reason for using these two wheat genotypes instead of the NILs in this experiment was that Sumai 3 has the strongest FHB resistance of all wheat genotypes that have been studied so far. It has not only *Qfhb1* but also other major FHB-resistance QTL, such as *Qfhb_6BL* (Basnet et al., 2012) and *Qhfs.ifa-5A* (aka. *Fhb5*, Buerstmayr *et al*., 2003; Lin *et al*., 2006; Xue *et al*., 2011), and it has been the most used

FHB resistance source in wheat improvement worldwide. *WFhb1-1* could be a major FHB-resistance gene if knocking it down can significantly compromise FHB resistance in Sumai 3. Y1193-06 has the worst FHB susceptibility of all wheat genotypes we have evaluated so far, and, thus, is the best control in contrast to Sumai 3.

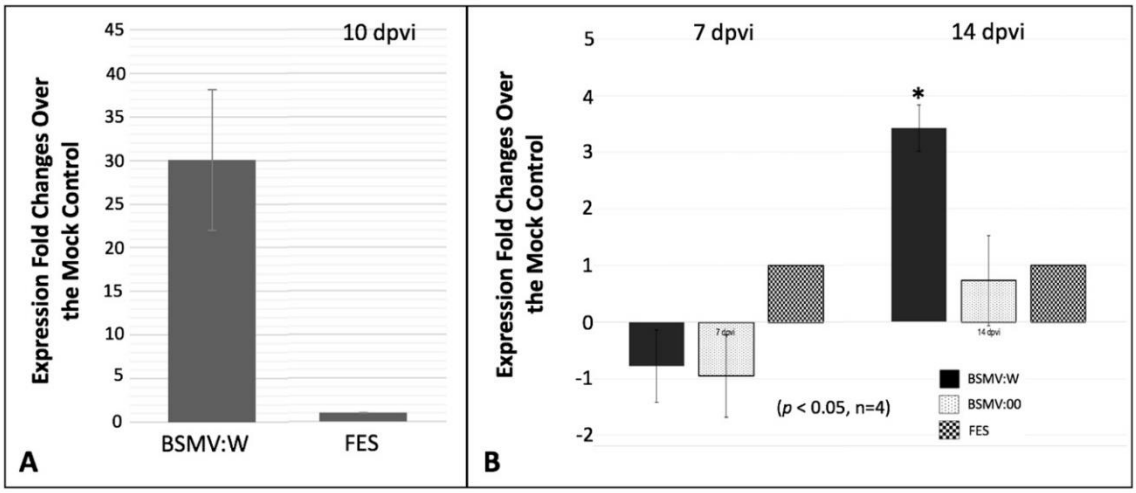


**Figure 2.4.** Results of RT-qPCR assays of *WFhb1-1* transcript abundance changes in leaves (**A**) or spikes (**B**) of the *Qfhb1*-NIL-S plants inoculated with the *WFhb1-1*-overexpressing strain (BSMV:W), wildtype BSMV (BSMV:00) in comparison with the viral inoculation buffer FES-inoculated mock control; dpvi: days post viral inoculation.

As we did in the *WFhb1-1* overexpression assay, we first tested if the knockdown works in Sumai 3. The leaves of Sumai 3 were inoculated with BSMV:T (the *WFhb1-1*-silencing BSMV strain), BSMV:00 or FES, and *WFhb1-1* expression was monitored at 7, 15 and 21 dpvi (Figure 2.7A). Significant down expression of *WFhb1-1* was observed at 15 dpvi in the BSMV:T-inoculated plants compared to those inoculated with BSMV:00 or FES. *WFhb1-1* mRNA abundance in the BSMV:T-inoculated plants was 59~97% less than in the BSMV:00-inoculated plants. *WFhb1-1* mRNA was 17.1~37.5% less abundant in the BSMV:00-inoculated plants than in the FES-inoculated plants, which is statistically

not significant ($p > 0.05$). As shown in Figure 2.7B, the *WFhb1-1*-silencing insert in BSMV:T seems to be partially deleted from its viral carrier probably during the viral replication. This could explain why we observed a down-fall in *WFhb1-1* silencing at 21 dpvi (Figure. 2.7A). Code optimization seems to be needed to make the insert stable.

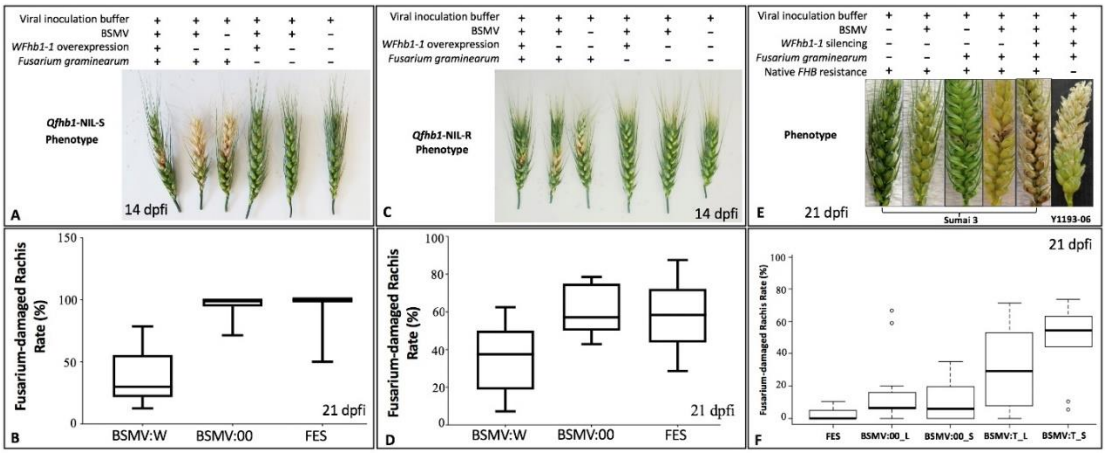


**Figure 2.5.** Photos and graphics showing the results of overexpression of *WFhb1-1* in near-isogenic lines *Qfhb1*-NIL-S (**A** & **B**) and *Qfhb1*-NIL-R (**C** & **D**) or silencing of *WFhb1-1* in FHB-resistant cultivar Sumai 3 (**E** & **F**) and FHB-susceptible landrace Y1193-06 (**E**) using a barley-stripe-mosaic virus (BSMV)-based system. Viral inoculation on spikes was applied. dpfi: days post *Fusarium* inoculation; FES: viral inoculation buffer FES; BSMV:00: wildtype BSMV; BSMV:W: *WFhb1-1*-overexpressing BSMV; BSMV:T: *WFhb1-1*-silencing BSMV; _L leaf inoculation of BSMV; _S: spike-inoculation of BSMV. The red or black dots on spikelets indicate the inoculated spikelets.

Then FHB inoculation was applied to the BSMV:T-inoculated and the control plants to test if silencing *WFhb1-1* in wheat spikes can increase FHB severity. In this experiment, BSMV:T was used for both leaf (this treatment was designated as BSMV:T_L) and spike (this treatment was designated as BSMV:T_S) inoculations in both Sumai 3 and Y1193-06. BSMV:00 and FES inoculations were conducted as the viral inoculation controls and

sterile water was used as the mock *Fusarium* inoculant. FDR was monitored at 7, 15, 21 and 28 dpfi. Our data show that FHB resistance is compromised in the BSMV:T-treated Sumai 3 plants (Figures 2.5E & 2.5F, Supplementary Figures S2.6 to S2.8), which enabled *F. graminearum* to quickly spread to adjacent spikelets, whereas, FHB symptoms in the FES- and the BSMV:00-treated Sumai 3 plants were mainly limited to the inoculated spikelets (Figure 2.5E; Supplementary Figure S2.8).

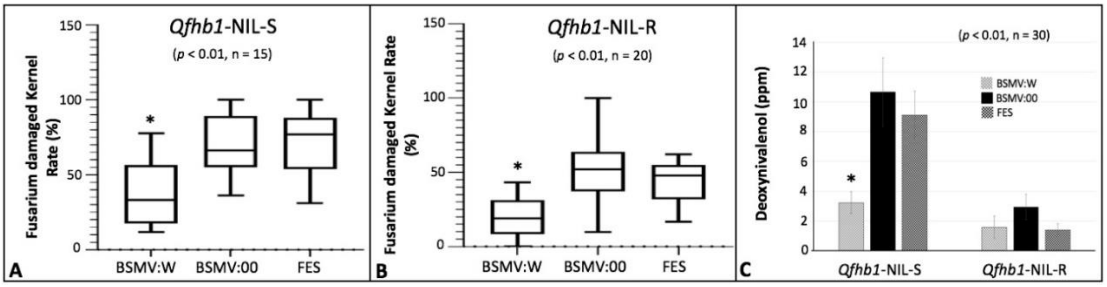


**Figure 2.6.** Graphics showing mean Fusarium-damaged kernel rate (%) (**A** & **B**) and deoxynivalenol content (ppm) (**C**) in the harvested kernels of *Qfhb1*-NIL-S and *Qfhb1*-NIL-R inoculated either with *WFhb1-1*-overexpressing BSMV:W, the wildtype BSMV:00 or the viral inoculation buffer FES. *: significantly different compared with others.

This successful breakdown of Type II resistance by *Fusarium* infection in BSMV:T-treated Sumai 3 plants could be phenotypically observed as early as 7 dpfi (Supplementary Figures S2.8). In these plants, the disease seemed to spread mainly toward the base of the spikes, with only one or two upper rachis internodes being infected. Even at 28 dpfi, the upper spikelets remained clear of the fungal infection (Supplementary Figure S2.8). As expected, no significant difference between treatments and control was observed in Y1193-06 (Figure 2.6E; Supplementary Figures S2.9 & S2.10). We noticed that the spikelets above the inoculation site on the BSMV:T+*Fusarium* inoculated spikes of Y1193-06 were dry to death (Figure 2.5E),

while this phenomenon was not observed on the BSMV:00+FHB inoculated Y1193-06 spikes. Therefore, knocking *WFhb1-1* down has enhanced FHB susceptibility in Y1193-06. Pearson's product-moment correlation test using mean value of *WFhb1-1* abundance at 24 hpfi and FDR index data at 7 dpfi, 15 dpfi and 21dpfi collected from Sumai 3 indicated a strong negative association between the two ($p$ = 0.00027 and *Cor* = -8.62). Our data indicate that viral inoculation on spike apparently is more effective in inducing RNAi than the leaf-inoculation (Supplementary Figures S2.6 & S2.7).

### 2.3.7 Evaluation of *WFhb1-1* expression in *TaHRC*-knockout Bobwhite mutant

CRISPR/Cas9-based gene editing of *TaHRC* in FHB-susceptible Bobwhite resulted in deletion mutations that knocked *TaHRC*, gaining FHB resistance (Su et al., 2019). Expression of *WFhb1-1*, *PFT*, *GDSL* and *TaHRC* were compared between the *TaHRC*-knockout mutant and the wildtype of Bobwhite. Compared to the wildtype Bobwhite, *TaHRC* expression was found ~2.5 or ~3.58 folds lower, respectively using the gene-specific primers reported by Su et al. (2019) or Li et al. (2019), in the spikelets of the knockout plants 24 hours after FHB inoculation, while the expression of *WFhb1-1* was ~3 folds higher in the knockout mutant plants. Whereas, in both Bobwhite lines, expression of *PFT* was undetectable and *GDSL* expression level was too low to make any meaningful analysis. Therefore, both *PFT* and *GDSL* do not seem to have any role in the FHB resistance conferred by *Qfhb1*.

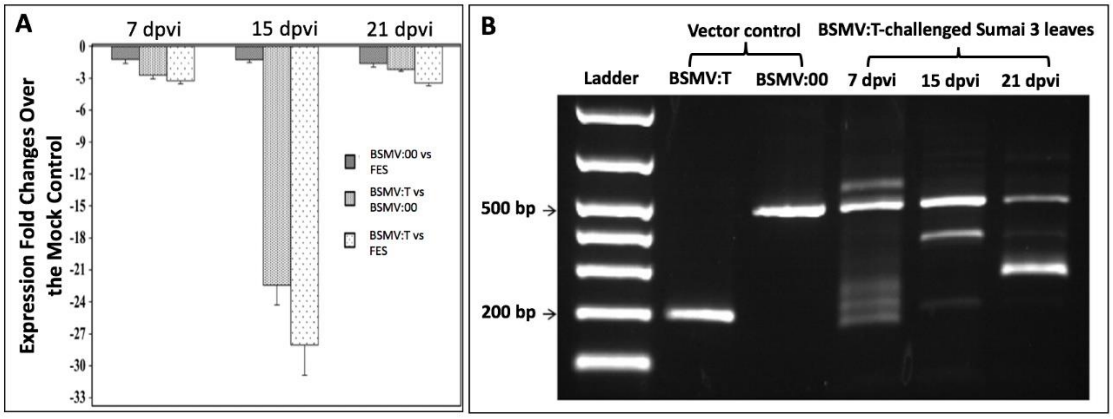


**Figure 2.7.** Results of RT-qPCR of *WFhb1-1* silencing (**A**) or PCR (**B**) assays of the stability of *WFhb1-1*-insert in BSMV:T viral vector in leaves of inoculated Sumai 3. Wildtype BSMV:00 and FES were used as the controls; dpvi: days post viral inoculation.

## 2.4 Discussion

The goal of this study is to identify the functional component of the major FHB resistance QTL *Qfhb1* in wheat. We have taken a functional approach to reach this goal. We first conducted transcriptomics analysis between FHB-resistant and FHB-susceptible wheat lines and identified 637 genes that have significantly changed their expression abundance in wheat spikelets after *F. graminearum* infection and, therefore, are FHB-related genes (Li and Yen, 2008). Of these 637 genes, 406 genes which are associated with FHB resistance were analyzed by expression QTL (eQTL) mapping, and the significantly changed expression of three genes were found to be associated with *Qfhb1* (Zhuang et al., 2013). Since these three genes can either physically locate in *Qfhb1* or physically locate at other places in the genome but are regulated by a gene in *Qfhb1*, we conducted a physical mapping using a series of nullisomic-tetrasomic lines of Chinese Spring to identify their physical locations in wheat genome and found that *WFhb1-1* is the only gene that is both physically located to wheat chromosome 3B where *Qfhb1*

locates and functionally associated with *Qfhb1*. In the present study, we have revealed the coding sequence of *WFhb1-1* (Figure 2.1), elucidated its expression at protein level (Figures 2.2 and 2.3), and transiently assayed its function in FHB resistance with overexpression and RNAi-induced silencing (Figures 2.4 and 2.5).

Data obtained in the present study shows that *F. graminearum* infection causes a significant reduction of *WFhb1-1* accumulation in NIL-S in the early hours of the disease development but not in NIL-R (Figure 2.3). This observation is in line with our previous observation at the transcription level showing negative regulation of *WFhb1-1* by the infecting pathogen. Our data have also shown that overexpressing *WFhb1-1* leads to a significant reduction in FHB severity under a high disease pressure in both the NILs, and the FHB resistance level in the *WFhb1-1*-overexpressing NIL-S plants is comparable to the level usually observed in NIL-R (Figures 2.5A to 2.5D, 2.6A, 2.6B; Supplementary Figure S2.5). By contrast, silencing *WFhb1-1* leads to the significant compromise of FHB resistance in Sumai 3 and a noticeably increased FHB susceptibility in Y1193-06 (Figures 2.5E & 2.5F; Supplementary Figures S2.6 to S2.10). Overexpressing *WFhb1-1* also significantly reduces DON content in the kernels of NIL-S (Figure 2.6C). Therefore, our findings show that *WFhb1-1* is a major FHB-resistance gene in wheat that can significantly reduce both FHB severity and DON accumulation even under high disease pressure. Wang et al. (2018) reported that *WFhb1-1* not only confers FHB resistance but also shows resistance to *Fusarium* root rot (FRR) by preventing the pathogen from spreading in the infected plant. It seems that *WFhb1-1* works in the whole plant to protect it against *Fusarium*-caused diseases.

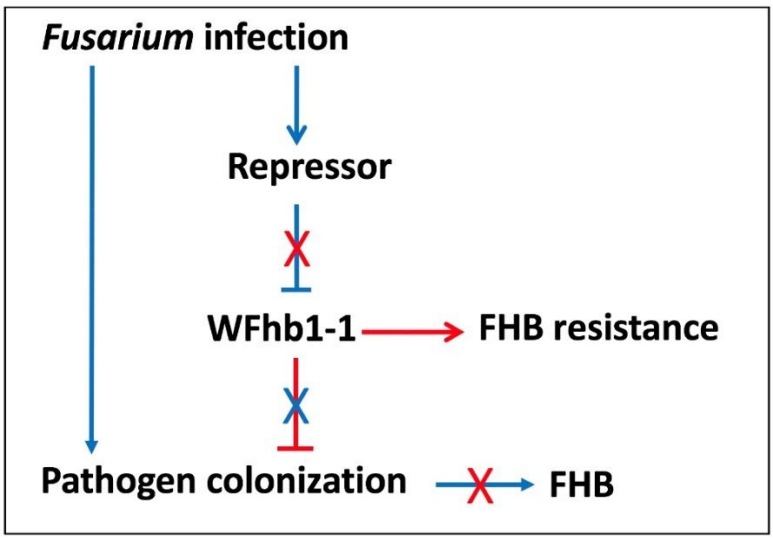


**Figure 2.8.** An illustration showing the hypothesis on how WFhb1-1 suppresses infecting pathogen leading to FHB resistance and how the infecting pathogen suppresses *WFhb1-1* expression to gain colonization and thus develop FHB on the host plant. Arrowhead lines indicate promotion, T-headed lines denotes suppression, X means interruption, blue lines/letter lead to FHB development and red line/letter results in FHB resistance.

Our data show that *P. pastoris*-expressed WFhb1-1 protein can inhibit growth of both *P. pastoris* and *F. graminearum* in culture (Figures 2.2D to 2.2G). These results suggest that WFhb1-1 is most likely an antifungal protein and probably functions to inhibit *F. graminearum* colonization *in planta*. Since our sequence analysis did not reveal any detoxification domain in WFhb1-1, the observed reduction in DON content in the kernels of the *WFhb1-1*-overexpressing NIL-S plants (Figure 2.6C; Supplementary Table S2.2) is probably due to a reduced fungal population on the plants by WFhb1-1's antifungal activity, not due to DON detoxification by WFhb1-1. It will be interesting to know if *WFhb1-1* has an even broader role in protecting wheat against more fungal diseases other than FHB and FRR.

Our study has confirmed our previous conclusion that *F. graminearum* can suppress

*WFhb1-1* expression in the early hours of FHB pathogenesis (Figure 2.3; Supplementary Table S2.1). This suppression may be a key step in FHB resistance. In a previous quantitative proteomic study of the same *Qfhb1* NILs, we found that FHB may result from a pathogen-promoted hypersensitive reaction (HSR) by the infected host cell and *Qfhb1* largely functions to either alleviate HSR or to manipulate the host cells to not respond to the pathogen promotion (Eldakak et al., 2018). Therefore, as illustrated in Figure 2.8, here we hypothesize a pathogen-host interaction model: *WFhb1-1* inhibits the growth of *F. graminearum* to prevent FHB development, the pathogen has developed an ability during the host-pathogen co-evolution to overcome *WFhb1-1*'s inhibition by suppressing its transcription during the initial stage of infection which leads to FHB development, and wheat then develops a currently unknown way to avoid the pathogen's suppression resulting in resistance to FHB again. In this model, we hypothesize that the pathogen may either produce and then deliver a *WFhb1-1* suppressor into the host or negatively regulate an indigenous host suppressor. In the first scenario, WFhb1-1 may work alone in contribution to FHB resistance. The second scenario actually fits to the multi-gene model of *Qfhb1*-confered FHB resistance proposed by Gao et al. (2005), Yang and Rajaram (1996), Schweiger et al. (2016) and Rawat et al. (2016). The pathogen's inability to suppress the *WFhb1-1* transcription from BSMV:W suggests that the suppression may target at the native *WFhb1-1* promotor or another regulation site but not *WFhb1-1* transcript itself. More research is needed to test this hypothesis and answer the question how FHB-resistant wheat genotypes escape this suppression.

Two recent publications (Su et al., 2019; Li et al., 2019) have associated *TaHRC* with the *Qfhb1*-confered FHB resistance. Although the resistance mechanisms reported in the

two reports are controversial, both concluded that *TaHRC* is an FHB-susceptibility gene and a deletion in this gene has caused the loss of the FHB susceptibility. Su et al. (2019) reported that *TaHRC* is a nuclear protein with an unknown biological function. Therefore, it could be a regulator to other functional genic components of *Qfhb1*. In the recent study, we have found that knocking *TaHRC* out in FHB-susceptible wheat cultivar Bobwhite by CRISPR/Cas9 gene editing also causes upregulation of *WFhb1-1*. This result indicates that *TaHRC* might negatively regulate *WFhb1-1* expression. Therefore, it is highly probable that TaHRC is the *WFhb1-1*-suppressor in our model presented in Figure 8, and in the cases reported by Su et al. (2019) or Li et al. (2019), a deletion in *TaHRC* causes the loss of its suppression of *WFhb1-1*, which results in a functional *WFhb1-1* during the pathogen infection and thus FHB resistance. Further researches should reveal how *TaHRC* regulates *WFhb1-1* and whether the pathogen indeed interacts with TaHRC to develop FHB.

As described by Zhuang et al. (2013), we previously identified *WFhb1-1* as a candidate of the functional genic component of *Qfhb1* on the basis of the following evidences: 1) *WFhb1-1* was differentially expressed between NIL-S and NIL-R early in the pathogenesis, which determines the FHB resistance or susceptibility in the NILs; 2) out of the 406 FHB-related genes investigated, *WFhb1-1* was the only one whose expression was significantly associated with *Qfhb1* by eQTL mapping, which suggests that *WFhb1-1* either physically locates in the QTL or is functionally controlled by a gene in this QTL; and 3) nulli-tetrasomic analysis has physically mapped it on chromosome arm 3BS. These arguments have been strengthened by the following findings from the present study: First, suppression of WFhb1-1 accumulation at the early stage of FHB

pathogenesis was observed in NIL-S but not in NIL-R (Figure 2.3); secondly, WFhb1-1 has been found to have antifungal ability (Figure. 2.2), thus, *WFhb1-1*'s role in FHB resistance seems to be an inhibitor to the pathogen growth *in planta*, which is well-aligned with *Qfhb1*'s function of conferring Type II resistance; and thirdly, FHB-susceptible NIL-S plants can be made as resistant as NIL-R plants are by simply overexpressing *WFhb1-1* in them, while silencing *WFhb1-1* in Sumai 3 resulted in complete compromise of FHB resistances (Figure. 2.5). However, the present study still does not give a conclusive answer to the question whether *WFhb1-1* is physically located in the *Qfhb1* interval or not. This is because *WFhb1-1* has been *in silico* mapped to a place between markers *Xsts9-2* and *Xsts65-3* in the 3BS pseudomolecule (Figure 2.1B), which is found to be within the *Qfhb1* interval defined by some (Basnet et al., 2012; Zhou et al., 2010; Zhuang et al., 2013) but outside the interval defined by others (Pumphrey, 2007; Rawat et al., 2016; Schweiger et al., 2016). Particularly, *WFhb1-1* is not among the genes in the QTL interval reported by Schweiger et al. (2016) or by Rawat et al. (2016). This controversy could be at least partially due to sequence diversity in the *Qfhb1*-containing region among wheat cultivars. In fact, Schweiger et al. (2016) reported a high dissimilarity between Sumai 3 and Chinese Spring in the core region of *Qfhb1* that hosts the FHB marker UMN10 and the three published *Qfhb1* candidate genes: *GDSL* (Schweiger et al., 2016), *PFT* (Rawat et al., 2016) and *HRC* (Su et al., 2018). Furthermore, several landmark markers of this QTL have inconsistent positions on 3BS among the wheat accessions used in different mapping studies, indicating occurrence of inversions among these wheat accessions. For example, marker *Xbarc147* is moved from the proximal side of marker *Xgwm533* in the Sumai 3/Stoa map by Liu et al. (2006) to the

distal side of *Xgwm533* in the Wangshuibai/Wheaton map by Yu et al. (2008) and the CS-SM3-3B/Annong8455 map by Zhou et al. (2010); *Xgwm493* is at the distal side of *Xumn10* in the Chinese Spring 3B pseudomolecule (Figure. 2.1B) but at its proximal side in the Sumai 3/Stoa map by Liu et al. (2006). Also, marker *Xbarc147* is at the distal side of *WFhb1-1* in our map (Zhuang et al., 2013) and the *Qfhb1* interval in the Sumai 3/Stoa map by Liu et al. (2006) but in the middle of *Qfhb1* in the Wangshuibai/Wheaton map by Yu et al. (2008). Furthermore, *PFT*, the proposed candidate gene of *Qfhb1* by Rawat et al. (2016), was in fact outside the QTL in FHB-resistant Wangshuibai and Sumai 3 according to Li et al. (2019). Therefore, it is possible that *WFhb1-1* and its regulator gene are both in the *Qfhb1* interval in some wheat lines but, in others, only the regulator is in the QTL with *WFhb1-1* itself flanking outside. Nevertheless, our data indicate that, being a genic component of *Qfhb1* or not, *WFhb1-1* is a key contributor to FHB resistance conferred by *Qfhb1* in wheat.

In summary, findings from this study demonstrate that *WFhb1-1* is a key FHB-resistance gene in wheat with antifungal function, and it most likely is a functional component of *Qfhb1* because *Qfhb1* cannot confer FHB resistance without *WFhb1-1*. Our data show that wheat needs a normal *WFhb1-1* expression at the initial stage of FHB pathogenesis to develop FHB resistance, while the pathogen needs to suppress *WFhb1-1* expression to colonize and thus cause FHB in wheat. Understanding how the pathogen suppresses *WFhb1-1* transcription will greatly help to our understanding of FHB pathogenesis and thus the control of FHB epidemics.

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# Chapter 3

## Fusarium graminearum virus-1 strain FgV1-SD4 infection eliminates mycotoxin Deoxynivalenol synthesis by *F. graminearum* in FHB.

### 3.1 Introduction

Mycoviruses are ubiquitous in nature, yet very few mycoviruses that exist are discovered (Cook et al., 2013; Marzano et al., 2016; Rosario and Breitbart, 2011). Fungal viruses are often associated with symptomless latent infections of their host; however, many mycoviruses are identified that reduce the virulence of fungal pathogens. Cryphonectria hypovirus 1 (CHV1), which infects and reduces the virulence of *Cryphonectria parasitica*, is one of the very few viruses, which has been utilized as a biological control agent (Ghabrial et al., 2015). Differences in vegetative compatibility is a major constraint in use of a capsidless mycoviruses like CHV1 since they are thought to spread primarily through fungal anastomosis and within spores (Choi et al., 2012). However, there are few reports of extracellular transmission of other mycoviruses suggesting that extracellular transmission of mycoviruses is possible (Marzano et al., 2015; Yu et al., 2013). A virus capable of extracellular transfection could potentiate the transmission of another capsidless virus by the process of transencapsidation, as reported in *Rosellinia necatrix* (Zhang et al., 2016). Moreover, coat protein (CP)-mediated transmission of plant viruses in the families Ophioviridae and Virgaviridae by internalization of virus particles within zoospores indicates the existence of different mechanisms of transmission of encapsidated viruses suggesting that it might be common in the nature (Rochon et al., 2004).

Fusarium graminearum virus 1 (FgV1) infects *Fusarium graminearum* and reduces its virulence. FgV1 was isolated and characterized over a decade ago (Yu et al., 2009; Kwon et al., 2007; Chu et al., 2002). This virus has gained interest for a potential of being used as a biological control agent against *F. graminearum* because this fungus is a notorious pathogen against small grain crops (Stack et al., 2003). However, there has been no report of successful development of protocol to utilize FgV1 as an effective biological control agent. FgV1 is a double-stranded RNA (dsRNA) virus, which is known to be transmitted intracellularly through spores and anastomosis. FgV1 has linear dsRNA genome with four ORFs, which is 6,621 bp long without its 3' poly (A) tail. Infection of *F. graminearum* by FgV1 has been associated with changes in various factors in the host, such as reduced mycelial growth, increased pigmentation, and reduced virulence on wheat plants and reduced levels of mycotoxin DON (Chu et al., 2002; Kwon et al., 2007; Yu et al., 2009). However, the underlying mechanism on how FgV1 downregulates DON biosynthesis is not clear.

A few previous studies showed that dsRNA-based gene silencing machinery can be induced in response to viral infections in *Neurospora crassa* and *Cryphonectria parasitica* (Cagoni and Macino, 1999; Zhang et al., 2008). In *F. graminearum*, *Dicer-2* and *Ago-1* proteins play critical role in small RNA induced silencing (Chen et al., 2015). Similarly, the role of *Dicer-like2* (*dcl2*) and *argonaute-like2* (*agl2*) as major RNAi players in this fungus is also reported (Galla, 2014; Lee et al., 2014; Andika et al., 2017). Yu et al. (2018) found that FgV1 infection interferes and downregulates *dcl2* and *ago1* to inhibit the antiviral defense mechanism of *F. graminearum*.

Since *F. graminearum* is one of the worst pathogens of small grain crops, viruses that can make it hypovirulent and reduce the level of DON are of keen interest for their potential application as biocontrol agents. FgV1 makes *F. graminearum* hypovirulent; however, hyphal anastomosis is the only method reported till date to introduce this virus into the virus-free *F. graminearum* strains (Yu et al., 2018). Developing an extra cellular transfection method for this virus to infect virus-free strains has a great scope for the ease of rapid transfer of the virus between different vegetative strains. Here, we share results of our efforts to transfer this virus extracellularly by protoplast transformation of virus-free *F. graminearum* and *F. oxysporum* and to explore how FgV1 infection downregulates DON biosynthesis in *F. graminearum*.

## 3.2 Materials and Methods

### 3.2.1 Plant and fungal materials

A pair of Near Isogenic Lines (NIL) of wheat carrying or not-carrying *Qfhb1* QTL, NIL 260-4-1-1-2 (NIL-R) and NIL-260-1-1-4 (NIL-S), and FHB susceptible Tibetan wheat landrace Y1193-6 were used in this study. NIL wheat seeds were developed and kindly provided by Dr. James Anderson's lab at University of Minnesota. *F. graminearum* isolates Fg-SD-4-1 (Fg-4-1 hereafter) and Fg-SD-4-2 (Fg-4-2 hereafter) derived from Fg-SD4 strain collected form Watertown, South Dakota, and another strain Fg-18-2 were used in this study. Fg-SD4 strain of *F. graminearum* was collected by Dr. Saukhat Ali's lab at South Dakota State University. *F. oxysporum* strain used was obtained from Dr. Shin-Yi Marzano's lab at South Dakota State University. For each experiment, at least 15 plants of each line per repeat per treatment were grown in pots filled with Miracle-Growth Potting Mix in a greenhouse or a growth chamber under a

16/8 h light/dark period, and 25/16°C day/night temperature, supplied with cool, white fluorescent lamps.

### 3.2.2 Identification and Confirmation of FgV1

After the identification of slow growing strain of *F. graminearum*, total RNA was extracted, libraries were prepared, and was sequenced using Illumina 2500 sequencer at Keck center in University of Illinois, Urbana-Champaign. Sequence reads were trimmed and assembled into contigs. Contigs with significant similarity to viral amino acid sequences retrieved from NCBI were identified, among which the sequence for FgV1 was identified. Sequences were assembled to get the whole genome sequence of FgV1 virus, which we named as FgV1 virus SD4 strain (FgV1-SD4). FgV1 genome specific primers were also designed based on the FgV1 genome sequence obtained. Presence of viral RNA genome in fungal hyphae was confirmed with RT-PCR.

### 3.2.3 Comparison of pathogenicity between Fg-4-1 and Fg-4-2

The two *F. graminearum* isolates derived from Fg-4 strain were used to compare the pathogenicity of the fungus having or not having the mycovirus FgV1, respectively, in wheat. Virus-free strain of *F. graminearum*, Fg-4-1, was obtained by single conidia germination method from the isolate Fg-4. Briefly, single spore was germinated in a single PDA plate from the original stock culture until a virus free growth was obtained. For the inoculation of fungus on wheat spikes, *F. graminearum* was cultured on carboxymethyl cellulose (CMC) medium for 4 days, and then spores were collected for plant inoculation. Previously described procedures for wheat spike inoculation by Li and Yen (2008) were used. Briefly, *F. graminearum* spores were filtered from CMC medium

through four layers of sterile cheesecloth. The tubes with filtrate were centrifuged at 3000 rpm for 10 minutes to settle the conidia as pellet and were re-suspended in required volume of sterile distilled water. The concentration of conidia was counted using a hemocytometer and adjusted to ~100,000 conidia/mL. The spikelet was challenged with 10 µL of water-suspension of *F. graminearum* conidia or sterile water only (as a mock control) at the stage when intensive yellow color of anthers was observed. For each treated spike, two first-flowering spikelets were inoculated to induce a disease pressure at the level that an FHB-resistant genotype will be maximally diseased at 28 dpfi (days post fungal inoculation). The inoculated spikes were immediately covered with plastic zip-lock bags with a wet cotton ball inside for 72 h, to maintain the optimal humidity and temperature to facilitate disease establishment.

For disease evaluation, FDR was calculated as percentage of diseased rachides of all rachides per spike and FDK was calculated as percentage of diseased kernels of all harvested kernels per spike. FDR data were averaged per time-point per treatment per experiment at 7, 14, 21 and 28 dpfi, respectively. Similar approach was taken to analyze FDK data per treatment. Total number of kernels per spike was also counted, averaged, and compared between three treatment groups. DON content in the harvested kernels per spike was measured by sending the harvested kernels to the DON testing lab at University of Minnesota and analyzed for each treatment in our lab.

### 3.2.4 Reverse transcription and RT-PCR

Total RNA was extracted from a pea sized hyphal mass of *F. graminearum* scraped from PDA plate with TRIZOL (Invitrogen) following the manufacturer's instructions. RNA quality was tested using 1% agarose gel and quantified using a

NanoDrop ND-1000 UV-Vis Spectrophotometer (Wilmington, DE). For each treatment, at least three biological replications were conducted. For reverse transcription, ~500 ng DNase I-treated total RNA was used for cDNA synthesis using GoScript™ Reverse Transcriptase system (Promega) with FgV1-SD4 specific reverse primer targeting 3' end of viral genome (Table 3.1). Synthesized cDNA was used to carry out PCR using Phusion high fidelity DNA polymerase master mix from Thermofisher.

For the RT-PCR reaction, 2X dilutions were made for reverse transcription products, and 1 µL diluted cDNA/20 µl reaction mixture was carried out with FgV1-F and FgV1-R primers using Phusion high fidelity DNA polymerase master mix with 2 min at 95°C, 35 cycles of 45 s at 95°C, 45 s at melting temperature, and 1 min at 72°C, and then 5 min at 72°C. Three technical replications were conducted.

### 3.2.5 Amplification of whole genome of FgV1-SD4 and cloning into ZeroBlunt plasmid vector

Whole FgV1-SD4 viral genome was amplified using the viral genome specific 5' and 3' primers (FgV1-F2 and FgV1-R2) and cloned into ZeroBlunt vector plasmid following the manufacturer's instructions. Cloning of the genome and its orientation was confirmed with band size in agarose gel, restriction digestion with enzymes, and sequencing.

### 3.2.6 In-vitro transcription of FgV1-SD4

Confirmed clones of FgV1-SD4 in ZeroBlunt plasmid vector were digested with *Not1* restriction digestion enzyme and in-vitro transcribed using mMessage mMachine T7 polymerase transcription kit following the kit protocol. Successful transcription was

confirmed by running the transcription product in 1% agarose gel. This transcription product was used for the transfection of protoplasts generated from *F. oxysporum* and *F. graminearum*.

### 3.2.7 Protoplast generation and transfection

The protocol previously described by Hallen-Adams et al. (2011) was adopted with modifications for protoplast generation of *F. oxysporum* and *F. graminearum*. We changed the enzyme concentration for drisilase and lysing enzyme to 12.5 mg/ml and 10 mg/ml, respectively, as recommended by Ramamoorthy et al. (2015).

For the transfection, 100 µl of protoplast suspension of $10^6$ protoplasts/ml density was mixed with 20 µg of viral transcript and was mixed in a tube. The mixture was then transferred into a pre-chilled 4 mm BioRad electroporation cuvette and incubated on ice for additional 5 minutes. After the incubation, the mixture was pulsed on BioRad Gene Pulser Xcell™ Electroporation System with 2.5 kV, c=25 µF and 200-ohm parameters. Immediately after the pulse, 750 µl of STC buffer was added in each cuvette and incubated on ice for 10 minutes. After incubation, 200 µL of this pulsed protoplast mixture was transferred at the center of an empty sterile Petri plate and 20 ml of regeneration medium was poured around it. Multiple plates were prepared for the whole transfected protoplast for the regeneration. After regeneration media solidifies, petri plates were incubated at 22$^o$ C for 7-10 days to regenerate the transfected protoplast.

After the regeneration of protoplast, areas of growth from regenerated plates showing different morphological characteristics were transferred to new PDA plates and sub-cultured for six successive generations. After these sub-cultures, transfected fungal

strains were tested for the presence of FgV1-SD viral genome by RT-PCR, as explained earlier. Assessment of pathogenicity of transfected new strains of *F. oxysporum* was carried out by plucking out same size of growth from wild type and transfected strain on PDA, and by applying those to ripen red tomatoes over a tiny single prick by a needle. For the controls, same size of PDA only was plucked out and was applied in the same way. Tomatoes that were inoculated with *F. oxysporum* were incubated at room temperature for a week and infection sizes were evaluated for all three treatments.

### 3.2.8 Statistical analyses

Data were analyzed using student t-test and one-way ANOVA.

## 3.3 Results

### 3.3.1 The discovery of Fg-4-2

We first observed that the *F. graminearum* isolate (now named as Fg-4-2) from strain Fg-SD4 became slow grower with more pinkish color and much less pathogenic than before.

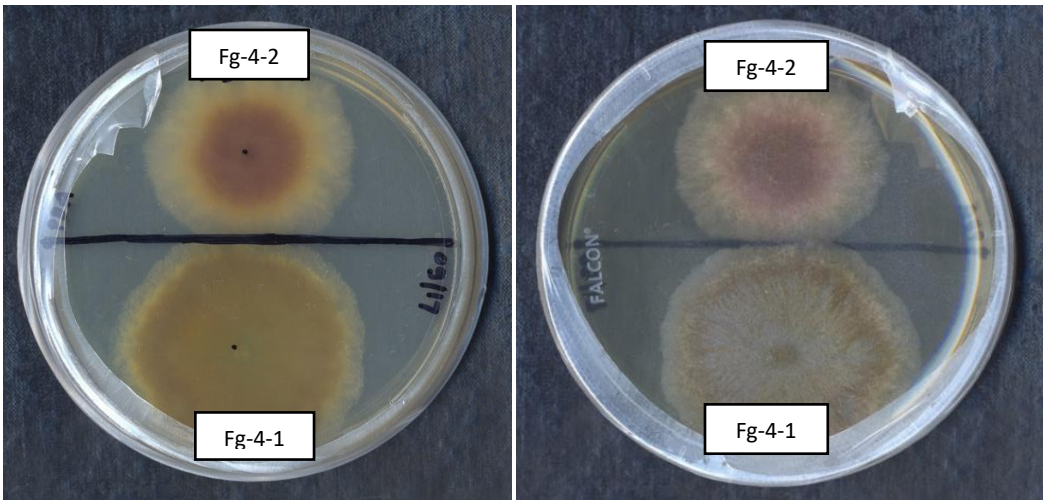


**Fig 3.1.** Comparison of growth of Fg-4-1 vs Fg-4-2 on PDA plates 5 days after inoculation

A comparison of this isolate with a freshly-made isolate from the original stock of the strain Fg-SD4 had confirmed the differences (Fig. 3.1). Therefore, the original strain was named Fg-4-1 and the new strain was named Fg-4-2. We noticed that Fg-4-2 produces more pigments and conidia than Fg-4-1 stain when the conidia were counted after four days of incubation. We suspected that mycovirus infection might be the cause of these difference.

### 3.3.2 Detection and confirmation of FgV1-SD4

RNA-seq analysis revealed that Fg-4-2 indeed contain a version of mycovirus FgV1. This was done by sequencing and analyzing the RNA libraries prepared from total RNA of Fg-4-2. The sequence tags obtained were blasted against viral database on NCBI and FgV1 genome was detected. The whole FgV1 sequence was assembled from the corresponding tags with the published sequence FgV1-DK3 in NCBI (Supplementary 3.1). The FgV1 revealed in Fg-4-2 was found ~96% identical to FgV1-DK3 sequence-wide, and thus is named FgV1-SD4. FgV1-SD4 genome specific primers were designed (Table 3.1) and used to detect and confirm the presence of FgV1-SD4 genome. Using FgV1-F and FgV1-R primers on cDNA synthesized using FgV1-R2 reverse primer and total RNA, presence of FgV1-SD4 genome was confirmed in Fg-4-2 strain of *F. graminearum* (Fig 3.2). We also designed primers for *Fusarium* Mitovirus (FgMV-F & FgMV-R) and tested with RT-PCR for its presence in any of the isolates but could not detect the genome. When we did the RT-PCR on cDNA from Fg-4-1 strain, we could not detect FgV1-SD4 virus (Fig 3.2). These results confirm that the morphological change observed in Fg-4-2 compared to Fg-4-1 was due to FgV1-SD4 infection of the latter.

**Table 3.1.** List of primers used in this chapter.

| **Primer name** | **Sequence** | **Purpose** |
|---|---|---|
| FgV1-F | GTTGCGTTGGAGGTTGACAC | RT-PCR |
| FgV1-R | CCAAAAACCACACGTCGTCC | RT-PCR, cDNA synthesis |
| FgV1-F2 | GGGGTATACTCTGATTATTTGAATTT | Viral genome cloning, Sequencing |
| FgV1-R2 | CATTTGGCCTCTAGACCCCCTATGCT | Viral genome cloning, Reverse transcription, Sequencing |
| FgMV-F | ACCATATCCCTTTTGGGGCTG | RT-PCR |
| FgMV-R | GTGCTCTTCCGATCTCCGTG | RT-PCR |

### 3.3.3 Pathogenicity in FHB

To confirm that the reduced pathogenicity of Fg-4-2 is actually caused by the FgV1-SD4 infection, the two strains of *F. graminearum* were, respectively, inoculated into spikes of FHB-susceptible wheat lines NIL-S and Y1193-06 to initiate FHB. Sterile water was used in the mock control treatment. The result shows that Fg-4-2 was indeed significantly hypovirulent compared to Fg-4-1 when the FHB severity were visually observed on the treated spikes (Fig 3.3).

Fusarium damaged rachides (FDR) percentage data was calculated at 7, 14, 21- and 28-days post *Fusarium* inoculation (dpfi) with either Fg-4-1, Fg-4-2 or sterile water on the flowering spikelets (Fig 3.4). Our result shows that FDR percentage was

significantly higher in the Fg-4-1-inoculated spikes compared to the Fg-4-2-inoculated spikes in all four time points analyzed. Similarly, Fusarium damaged kernels (FDK) percentage and total number of kernels harvested per spike were also analyzed (Fig 3.5). FDK percentage was also found to be significantly higher in the Fg-4-1-inoculated spikes compared to the Fg-4-2-inoculated ones. Additionally, Number of kernels per spike was significantly lower in the Fg-4-1-inoculated spikes compared to the Fg-4-2-inoculated spikes (Fig 3.5).

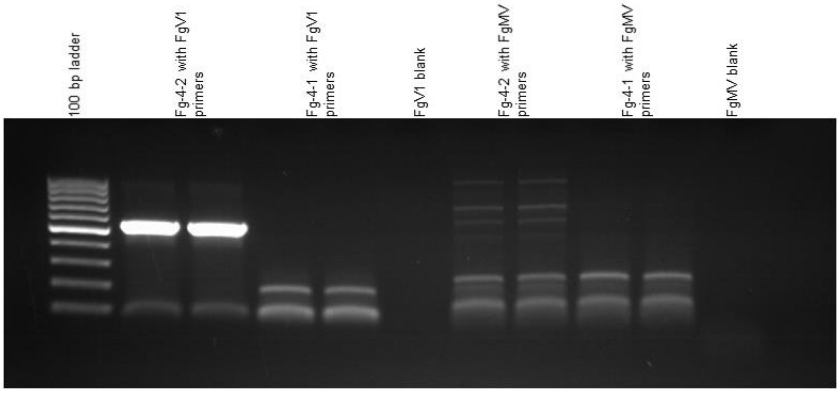


**Figure 3.2.** Gel image conforming the amplification of FgV1 viral genome in Fg-4-2 isolate of *F. graminearum*.

Mycotoxin Deoxynivalenol (DON) content was also compared between kernels harvested from spikes of wheat NIL-S inoculated with Fg-4-1, Fg-4-2 and sterile water. Interestingly, no DON was detected in kernels from spikes that were inoculated with Fg-4-2, and the data was similar to water inoculated treatment. However, there was ~18 ppm DON on seeds harvested from spikes inoculated with, Fg-4-2, the *F. graminearum* strain not having FgV1 (Fig 3.6).


**Figure 3.3.** Photos showing typic symptoms of FHB on spikes of wheat genotype NIL-S (A to F) and Y1193-06 (G to I) one (in case of Y1193-06) or two weeks (in case of NIL-S) post inoculation with the *F. graminearium* isolate Fg-4-1 (**A, B and G**), Fg-4-2 (**C, D and H**) or water (**E, F and I**). Red spots indicate the inoculated spikelets.

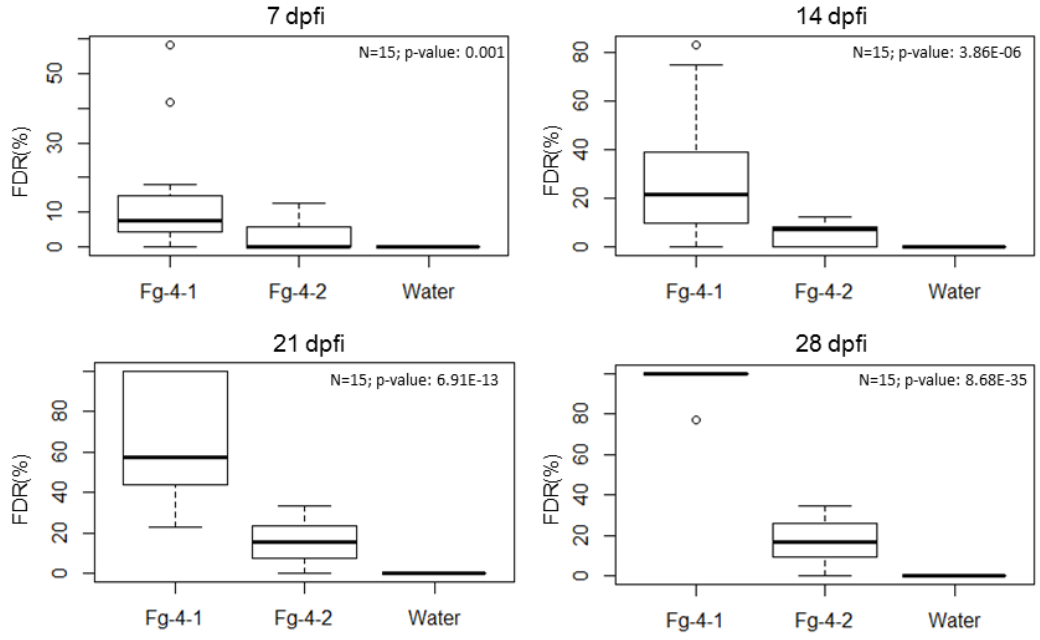


**Figure 3.4.** Mean FHB damaged rachises rates (FDR) percentage after inoculation of wheat line NIL-S with *F. graminearum* isolates Fg-4-1, Fg-4-2, and sterile water in 7, 14, 21 and 28 dpfi.

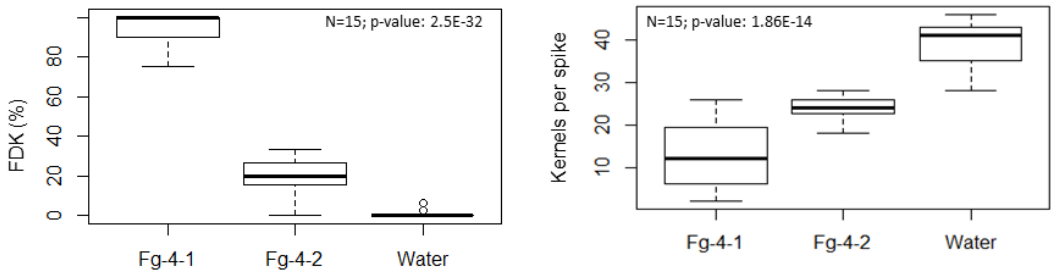


**Figure 3.5** Mean FHB damaged kernels rates (FDK) (left) and total kernels per spike (right) after inoculation of wheat NIL-S with *F. graminearum* isolates Fg-4-1 and Fg-4-2, and sterile water.

Clearly, these results demonstrate that Fg-4-2 is significantly hypovirulent compared to Fg-4-1. FgV1-SD4 has indeed reduced the pathogenicity of Fg-4-2 significantly.

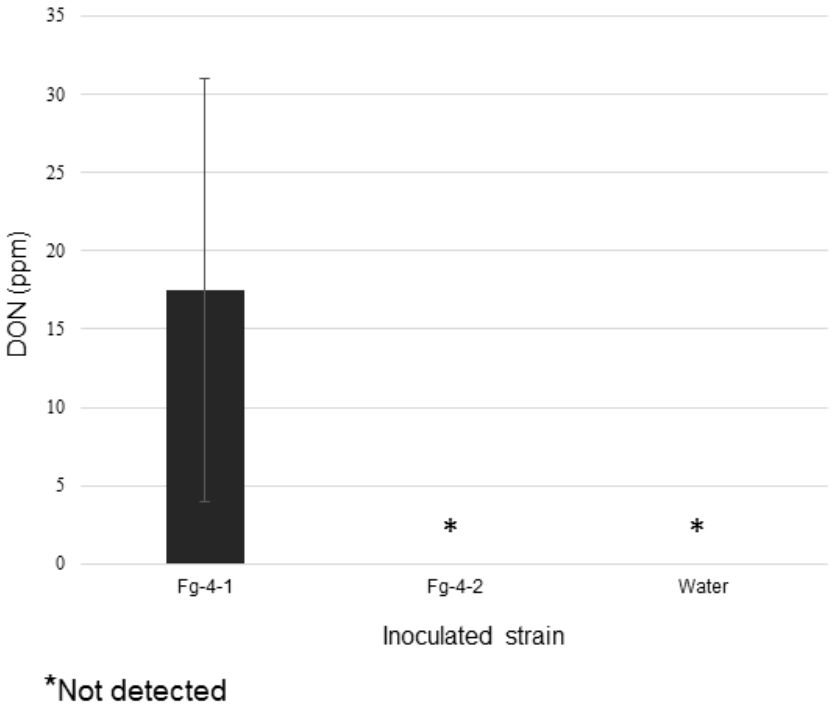


**Figure 3.6.** Deoxynivalenol (DON) toxin accumulation after inoculation of wheat line NIL-S with *F. graminearum* isolates Fg-4-1 and Fg-4-2, and sterile water.

### 3.3.4 Transmission of FgV1

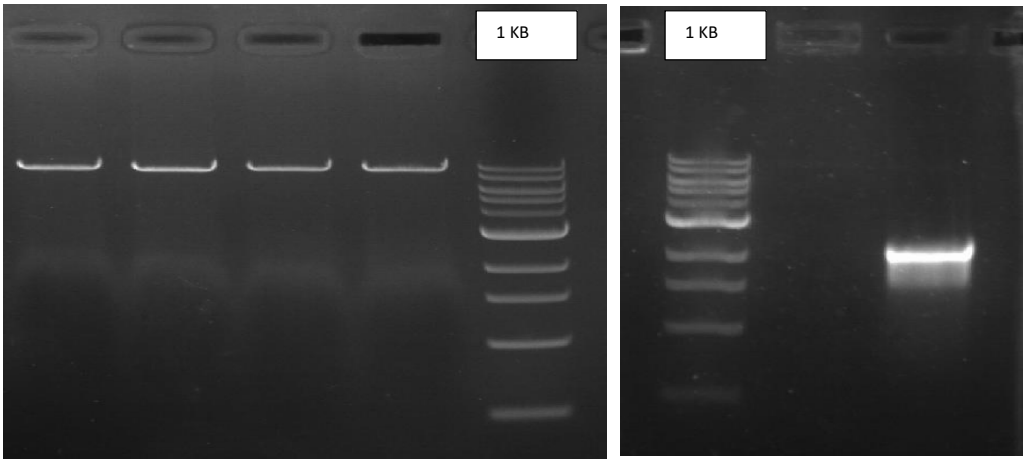


**Figure 3.7.** Gel pictures of linearized ZeroBlunt plasmid using BamHI restriction site (on left), and transcription of FgV1 genome using T7 polymerase (on right).

FgV1 is known to be transmitted in intracellular ways horizontally (between compatible hyphae by anastomosis) or vertically through conidia or ascospores. There is no report yet about extracellular transmission of this virus. Therefore, we conducted experiment to see if FgV1-SD4 is extracellularly transmissible or not. Specifically, we tried to inoculate FgV1-SD4 to virus-free strains of *F. graminearum* and *F. oxysporum* by protoplast transfection method. For this purpose, the whole genomic sequence of FgV1-SD4 was amplified by RT-PCR and cloned into ZeroBlunt plasmid vector. The subsequent DNA sequence for the viral genome was then successfully transcribed *in vitro* to get the whole sequence of the viral genomic RNA (Fig 3.7).

Protoplasts of both *F. graminearum* and *F. oxysporum* with concentration ~$10^6$/ml were made, and transfection of them was carried out using the *in-vitro* transcribed RNA. Although, the transfected strains did show some morphological differences in

pigmentation (Fig 3.8), we failed to detect the presence of FgV1-SD4 with RT-PCR in the transfected strains after six successive subcultures.

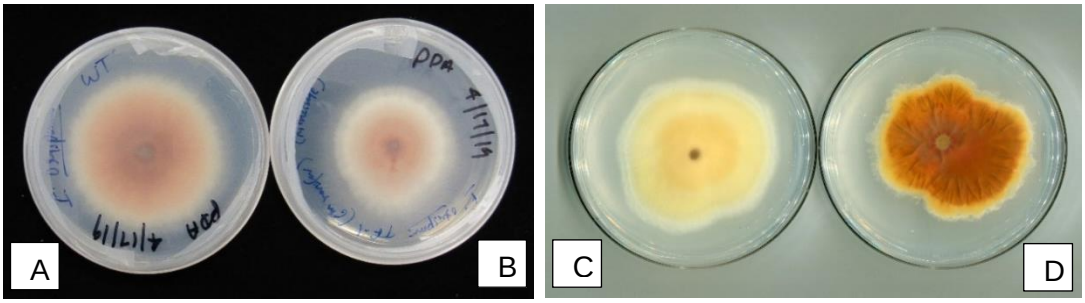


**Figure 3.8.** Phenotypes of cultured *F. oxysporum* and *F. graminearum* strains. **A**: *F. oxysporum* WT; **B**: *F. oxysporum* transfected; **C:** *F. graminearum* (Fg-18-2) WT; **D**: *F. graminearum* (Fg-18-2) transfected, respectively.

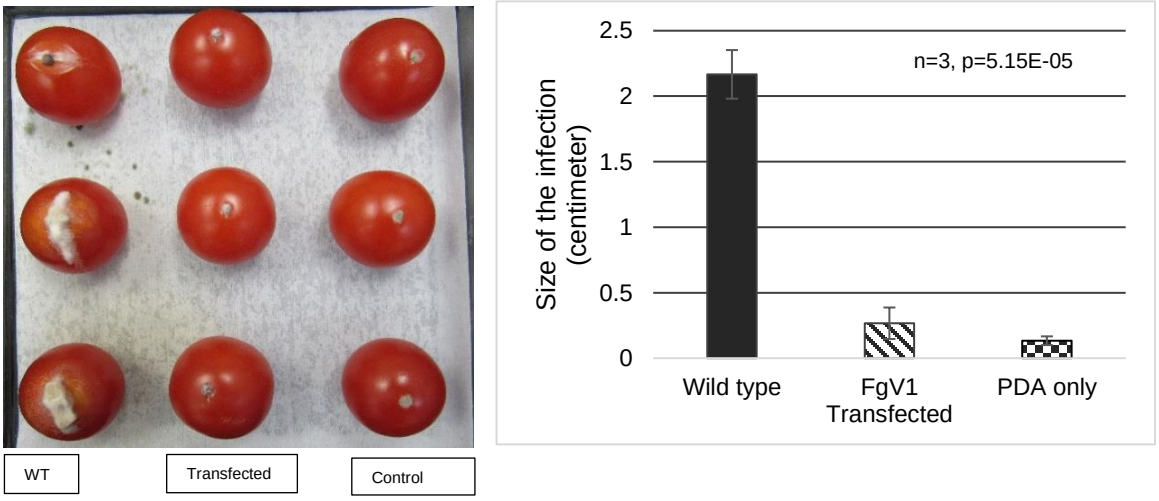


**Figure 3.9.** Left: Disease phenotypes of tomatoes a week after the inoculation of *F. oxysporum* (Transfected vs WT); PDA only was used as the mock control. Right: Analysis of disease development.

To compare the pathogenicity of transfected *F. oxysporum* with the wild type, ripe tomatoes were inoculated with equal size of fungus growth plucked from PDA plates. Disease progression on tomatoes were evaluated after a week of inoculation, and the infection size was measured for each individual tomato. As shown in Figure 3.9, the

infection size for the tomatoes inoculated with wildtype *F. oxysporum* was significantly higher compared to the tomatoes that were inoculated with transfected *F. oxysporum* or the control group tomatoes.

### 3.4 Discussion

In this study, we confirmed the existence of FgV1-SD4 in *F. graminearum* isolate Fg-4-2. More importantly, we validated its role in hypovirulence of *F. graminearum* and *F. oxysporum*, and in eliminating the accumulation of mycotoxin DON in the infected grains. The observed total elimination of DON from wheat grains infected by *F. graminearum* strain carrying FgV1-SD4 is very interesting, and promising for the use of this mycovirus as a potential biocontrol agent. We could also recover virus free strain of *F. graminearum* from Fg-4 original culture by single conidia culture method. Here, strain Fg-4 might have had very low titer of FgV1-SD4 virus but when it was sub-cultured multiple times, virus may have transfected almost all of the cells thus making the strain Fg-4-2 hypovirulent compared to the original stock.

RNAi mediated defense against viral pathogens is well conserved in eukaryotic life, and it is also present in many fungi (Andika et al., 2018; Campo et al., 2016; Segers et al., 2007). The RNA silencing response against viruses is well studied in *C. parasitica* against CHV1 and involves the induction of *dcl2* and *agl2* transcripts, and production of hairpin RNA (hpRNA). On the other hand, CHV1 infection of *C. parasitica*, suppresses RNA silencing mechanism in the fungus through a suppressor protein known as p29, which inhibits the upregulation of *dcl2* and *agl2* in the host to counter the host's defense responses (Segers et al., 2006; Segers et al., 2007; Zhang et al., 2008; Sun et al., 2009). In *F. graminearum Ago-1* and *Dicer-2* have critical role in hpRNA-mediated gene silencing,

and that *Dicer-2* also plays a role in miRNA-like small RNA (milRNA) generation (Chen et al., 2015). Expression of *Dicer-1* protein was increased but expression of *Dicer-2* and *Ago-1* proteins were significantly decreased following FgV1 infection of the *F. graminearum* (Lee et al., 2014; Yu et al., 2018). Similarly, *Ago-1* overexpressed *F. graminearum* mutant, after FgV1 inoculation showed significantly higher mycelial growth compared to the wild type after FgV1 infection. Same *Ago-1* overexpression mutant also showed significantly less viral dsRNA accumulation compared to the wild type. This shows that *F. graminearum* uses its RNA silencing machinery to silence RNA viruses including FgV1. However, FgV1 overcomes this RNA silencing machinery and further suppresses RNAi pathways of the host themselves via suppression of *Dicer-2, Ago-1* and, *dcl2* in *F. graminearum* to establish its infection in the host by interfering with host's antiviral response (Yu et al., 2018).

In the previous studies from our lab, we found that *dcl2* knockdown mutant of *F. graminearum* showed less virulence and significantly less accumulation of DON (Galla, 2014). We also found that this suppression of DON biosynthesis is probably mediated by siRNA *fg*siR34. DON biosynthesis is regulated by *Tri* genes present in three clusters in three different chromosomes of *F. graminearum* genome. Deletion/knockdown mutant studies have shown that *Tri5*, *Tri4*, *Tri14* are the major genes involved DON biosynthesis where as *Tri6* and *Tri10* are the major regulatory genes. Our studies show that expression of *Tri5* and *Tri6* genes is at least partially regulated by siRNA *fg*siR34 (Galla, 2014; Dahal, 2016). *Tri5* gene encodes an enzyme that catalyzes the first step of DON biosynthesis, and *Tri5* is controlled by *Tri6*. A potential target site that matches with partial sequence of *fg*siR34 between *Tri6* and *Tri5* gene in the cassette of *Tri* genes in *F.*

*graminearum* also suggests a regulatory role of this small RNA in *Tri* genes. Here, we propose a model of DON biosynthesis reduction due to FgV1-SD4 infection in *F. graminearum* (Fig 3.10). We propose that expression of *fg*siR34 in *F. graminearum* is indirectly suppressed by FgV1-SD4 through suppression of *Ago1/Dicer-2/dcl2* by the virus. This ultimately results in decreased biosynthesis of DON through downregulation of *Tri* genes in FHB as *fg*siR34 regulates the expression of *Tri6/Tri5* genes.

Although electroporation of *in vitro* transcript into the protoplasts has changed the phenotypes and pathogenicity of the transfected *F. oxysporum* and *F. graminearum* strains (Figures 3.8 and 3.9), we could not confirm the existence of FgV1-SD4 in these two transfected fungi by RT-PCR assay. This result could mean that the virus might need poly-A tailed transcripts to initiate replication or modifications in the method of transfection. Vegetative incompatibility is a barrier for most of the mycovirus transmission. However, novel approaches such as “super donor” with all the vic genes disrupted (Zhang and Nuss, 2016) should be developed to try to transmit this virus into virus-free strains of fungi due to the great potential of this virus as a biocontrol agent.

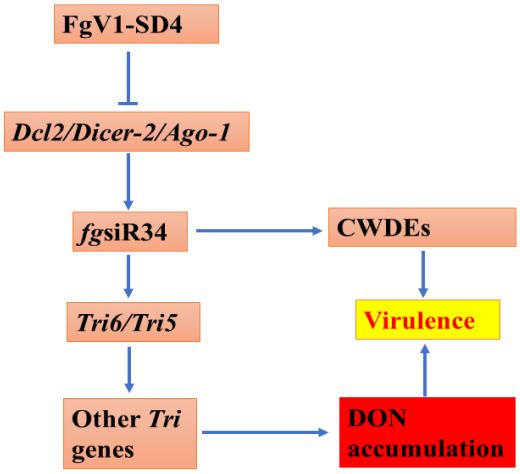


**Figure 3.10** Proposed model of DON regulation in *F. graminearum* by FgV1 infection.

## 3.5 Acknowledgement

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# Chapter 4

## Barley Stripe Mosaic Virus Induced Overexpression of gene has a potential of transmission to next generation through seeds in Wheat

### 4.1 Introduction

Functional characterization of genes is an important aspect of molecular study of organisms, and the manipulation of these genes to make the organism more adapted for changing environment. In agriculture, functional characterization and validation of genes is vital to develop cultivars better adapted to environment, greater yield and having various disease resistant traits to meet the ever-increasing demands of food supply (Vain, 2007). Development of transgenic plants through *Agrobacterium* mediated or biolistic method of transformation is the primarily tool for external gene integration into genomes of several crop plants (Vasil et al., 1992; Cheng et al., 1997; Barampuram and Zhang, 2011). These are the approaches that has been used for a long time to overexpress an external gene in a plants. More recently, the same approaches are modified for adapting the techniques for CRISPR/Cas9-based gene editing (Zhang et al., 2016; Cao et al., 2016; Zhang et al., 2019). Other systems like electroporation, microinjection, silicon carbide and chloroplast transformation are also used to deliver a gene into plants (Rakoczy-Trojanowaska, 2002). However, *Agrobacterium* mediated, and biolistic method of transformation are still the most commonly used methods (Barampuram and Zhang, 2011). These methods of plant transformation have two major drawbacks. First, they have very low efficiency of successful transformation and regeneration, and the processes are labor and cost intensive. Second, these techniques require many regulatory hurdles

related to transgenic plants because of stable integration of foreign genes into crop plants genome. Therefore, overexpression or silencing of genes in crop plants cannot be considered as a routine practice by biologists and breeders yet. A robust and rapid system to evaluate gene function in a biological system has a huge potential to validate the function of genes for their use in stable transformation, transgenic crops, and breeding programs.

Plant viruses are alternative tools to stable transformation. They can be used to express heterologous proteins, a foreign gene or a non-coding sequence throughout the plant in a very short time (Hefferon, 2014). *Tobacco mosaic virus*, *Potato virus X*, and *Tobacco rattle virus* are the most widely and successfully used viruses in plants for this purpose (Chapman et al., 1992; Baulcombe et al., 1995; Jia et al., 2003; Lu et al., 2003; Hefferon, 2014). However, there are very limited number of viral vectors for monocot plants, which are more recalcitrant for transformation. In recent years, barley stripe mosaic virus (BSMV) has been used and been modified to adapt to the monocot system, primarily for gene silencing studies (Purkayastha and Dasgupta, 2009; Senthil-Kumar and Mysore, 2011; Yuan et al., 2011; Cheuk and Houde, 2017; Cheuk and Houde, 2019).

BSMV is a positive sense, single-stranded RNA virus. It typically has tripartite genome with three parts (α, β and γ RNAs). RNAβ encodes coat protein and movement proteins, whereas RNAα and RNAγ play role in viral genome replication (Petty et al., 1990; Lee et al., 2012). cDNAs of these three components of BSMV genome has been cloned into three plasmids for the ease of propagation and in-vitro transcription to use them for gene expression studies (Jackson et al., 2009; Pacak et al., 2010; Yuan et al., 2011 Jian et al., 2017). Recently, a four component BSMV plasmid system has been

developed to carry relatively larger cargo size (cDNA of upto 2100 nucleotides) for overexpression (VOX) of genes (Cheuk and Houde, 2018). However, there still have been very few reports of successful use of BSMV-based VOX. The BSMV-mediated VOX is known to function only transiently. It has been known for a long time that BSMV can be transmitted to next generation through seeds (McKinney and Greeley, 1965); however, there is no report of whether the BSMV-mediated VOX is also transmissible to the next generation. Similarly, systematic studies about the rate of BSMV transmission in next generation through seeds and its effects in quantity of grains harvested are lacking. Here, we report a successful transmission of the BSMV-mediated VOX of *WFhb1-1* gene in T1 generation of wheat, when the three BSMV plasmid VOX system was applied in T0 generation.

## 4.2 Materials and Methods

### 4.2.1 Plant materials and growth

A pair of Near Isogenic Lines (NIL), NIL 260-4-1-1-2 (NIL-R) and NIL-260-1-1-4 (NIL-S) of wheat carrying or not carrying *Qfhb1* QTL, were used in this study. NIL wheat seeds were developed and kindly provided by Dr. James Anderson's lab at University of Minnesota. For each experiment, at least 10 plants of each line per repeat per treatment were grown in pots filled with Miracle-Growth Potting Mix in a greenhouse or a growth chamber under a 16/8 h light/dark period, and 25/16°C day/night temperature, supplied with cool, white fluorescent lamps. Plants were fertilized every week.

### 4.2.2 Construction of BSMV vector

The ORF sequence for *WFhb1-1* was synthesized according to the cloned sequence with EcoRI site on both 5' and 3' ends. The synthesized foreign gene expression insert was put into *pUC57* and cloned into *Escherichia coli* JM109 competent cells by heat shock method. Briefly, 1 μg pUC57_insert plasmid was added to 50 μl *E. coli* cells. The mixture was incubated on ice for 20 min, heat-shocked at 42°C for 50 s and immediately kept on ice for 2 min. Then, 950 μl SOC medium was added into mixture followed by ~1.5 h incubation at 37°C with shaking at ~150 rpm. The culture (50 μl and 100 μl) was spread on LB agar plates (with ampicillin 100 μg per ml) in duplicate. The plates were incubated at 37°C overnight and plasmid DNA was extracted from developed colonies for cloning into γ plasmid of BSMV.

The three plasmid BSMV system used in this study was kindly provided by Dr. Huang Li at Montana State University, Bozeman, USA. The BSMV γ vector (γ PCR vector) was modified by Huang's group with two *Xcm I* restriction sites inserted. We further modified it by adding an *EcoR I* site between the two *Xcm I* sites. To construct the overexpression vector BSMV:W, *WFhb1-1*'s (384 bp) with a EcoRI site *pUC57* plasmid was digested with *EcoR I* and the ORF sequence was released. BSMV:W was then constructed by digesting the γ-PCR vector and mixed then together to have the *WFhb1-1* ORF inserted in the EcoRI site of γ-PCR vector. The desired orientation of the ORF was confirmed with PCR using *WFhb1-1*-ORF forward/Gamma-1 reverse primers.

### 4.2.3 In-vitro transcription of viral RNA and plant inoculation

The three BSMV RNA chromosomes were reversely transcribed from the corresponding BSMV vectors following the protocol provided by Dr. Li Huang of Montana State University. RNA quality was assessed on 1% agarose gel. Virus

inoculation was done by following the previously described inoculation procedures (Ma et al., 2012; Scofield et al., 2005). Briefly, a mixture of the three viral RNAs in viral inoculation buffer FES was manually rubbed on 2$^{nd}$ leaf of 10 days old wheat seedlings. Viral inoculation was carried out with either the VOX vector BSMV:W or the viral inoculation buffer FES alone on at least 10 plants per line per treatment.

### 4.2.4 Screening of plants for BSMV assembly and *WFhb1-1* overexpression evaluation

Total RNA was extracted from leaf with TRIZOL (Invitrogen) following the manufacturer's instructions. RNA quality was tested using 1% agarose gel and quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Wilmington, DE). For each treatment, at least three biological replications were conducted. For reverse transcription, ~500 ng DNase I-treated total RNA was used for cDNA synthesis using GoScript™ Reverse Transcriptase system (Promega) with oligo(dT)15 primer. The presence of BSMV viral genome in the cDNA samples was confirmed before RT-qPCR was conducted. The RT-qPCR was conducted on QuantStudio 6 Flex (Applied Biosystems). Briefly, 2X dilutions were made for reverse transcription products, and 1μL diluted cDNA/20μl reaction was carried out using SYBR green I master Mix with 2 min at 95°C, 45 cycles of 20 s at 95°C, 30s at melting temperature, and 30 s at 72°C, and then 5 min at 72°C. Wheat β-actin gene was used as an internal control to normalize the Ct value. For each sample, three technical replications were conducted. Fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### 4.2.5 Fungal inoculum preparation and inoculation

*F. graminearum* isolate Fg-4-1, collected from Watertown, SD was used in this study to induce FHB. *F. graminearum* was cultured on potato-dextrose-agar (PDA) medium for a week, and then spores were collected for plant inoculation. Procedures used for wheat spike inoculation as previously described (Li and Yen, 2008). Briefly, *F. graminearum* spores were washed from PDA plates using sterile water and then filtered through four layers of sterile cheesecloth. The concentration of conidia was counted using a hemocytometer and adjusted with sterile water to about 100,000 conidia/mL. The spikelet was challenged with 10 μL of water-suspension of *F. graminearum* conidia or sterile water alone (as a mock control) at the stage when intensive yellow color of anthers was observed. For each treated spike, the two first-flowering spikelets were inoculated to introduce a high disease pressure. The inoculated spikes were immediately covered with plastic zip-lock bags with a wet cotton ball inside for 72 h to maintain the optimal humidity and temperature to facilitate disease establishment.

### 4.2.6 FHB evaluation between *WFhb1-1* overexpressed and wild type plants

For disease evaluation, FDR was calculated as percentage of diseased rachides of all rachides per spike and FDK was calculated as percentage of diseased kernels of all harvested kernels per spike. FDR and FDK were averaged per time-point per treatment per experiment at 7, 14, 21 and 28 dpfi, respectively.

### 4.2.7 Collection of seeds for T0 and germination of T1 plants

All the seeds from BSMV confirmed positive plants that were not inoculated with *F. graminearum* were collected and stored at $4^{o}$ C for a month. After that, the seeds were

germinated and grown in greenhouse as explained earlier to get the T1 generation of plants.

### 4.2.8 Screening of BSMV in T1 plants

Total RNA was extracted from T1 plants and cDNA was synthesized as explained above and the presence of BSMV was confirmed by RT-PCR using BSMV specific forward and reverse primers (Gamma-1-F and Gamma-1-R) (Table 2.1). Presence of viral genome was correlated with viral symptoms on leaves and T1 plants were categorized into two groups: one having BSMV virus and another not having BSMV virus.

### 4.2.9 Evaluation of *WFhb1-1* expression and pathogenicity of *F. graminearum* in T1 plants

Expression of *WFhb1-1* was also evaluated by qPCR between the two groups, BSMV+ (having BSMV) and BSMV- (not having BSMV), of T1 plants, by collecting three random samples from each group. Disease evaluation was done with FDR and FDK data collection, as explained above for T0 plants.

### 4.2.10 Evaluation of seed harvesting in T1 plants

Data for Number of tiller/spikes and total number of seeds per plant were collected and evaluated between two groups of T1 plants i.e. having or not having BSMV, without inoculation by *F. graminearum*.

### 4.2.11 Statistical analysis

Single factor ANOVA and Student's T test were used to analyze the collected data.

## 4.3 Results

### 4.3.1 BSMV inoculation and Overexpression of *WFhb1-1* in NIL-260-4 wheat

*WFhb1-1* gene confers FHB resistance in wheat (Zhuang 2013; Chapter 2). To understand if this resistance is passed down to T1 generation through seeds when *WFhb1-1* is overexpressed using BSMV-based VOX system, we applied this system to NIL-S to see how overexpression of *WFhb1-1* will impact FHB susceptibility/resistance. The second leaves of 10-day old wheat seedlings were inoculated with either *WFhb1-1*-overexpressing BSMV strain, BSMV:W, or FES viral inoculation buffer. We first examined the existence of BSMV:W and the *WFhb1-1* overexpression by RT-qPCR in newly emerged leaves of the inoculated plants at 10 dpvi (days post viral inoculation). The BSMV was successfully detected in 4 out of 20 BSMV:W-inoculated wheat plants but not in the FES-inoculated plants by RT-PCR, indicating that the infected BSMV:W was successfully assembled in 20% of inoculated plants within the infected wheat leaves and were able to move to other parts of the plants. We detected up to ~30 fold increase in *WFhb1-1* expression in BSMV:W-inoculated NIL-S plants compared to the FEF-inoculated NIL-S plants (Chapter 2). This observation confirms that BSMV:W infection can induce overexpression of *WFhb1-1* in the infected NIL-S plants.

Four BSMV+ *WFhb1-1* overexpressed plants, and four FES inoculated (BSMV-) four plants (T0 generation) were grown to mature, and seeds were harvested from each plant to grow the next generation of plants (T1 generation).

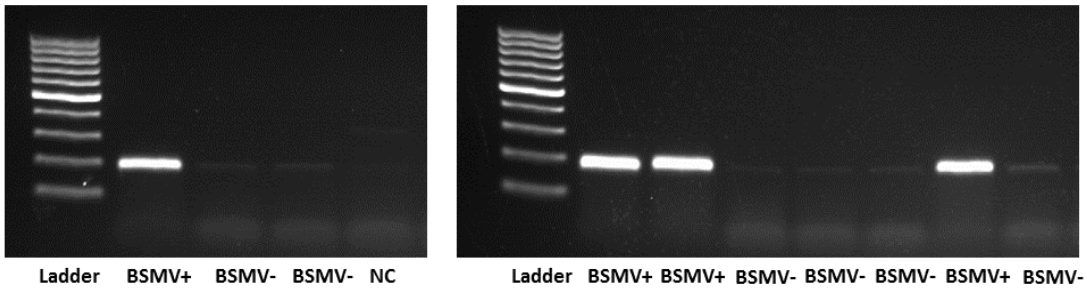


**Figure 4.1.** BSMV specific PCR for cDNA samples extracted from T1 generation of wheat plants having or not having BSMV virus. T1 generation plants were harvested from BSMV:W confirmed parent. BSMV+ means BSMV virus present. BSMV- means BSMV virus absent. NC means negative control.

### 4.3.2 BSMV transmission in T1 generation through seeds and overexpression of *WFhb1-1*

In T1 generation, among 53 offspring germinated from the four BSMV-confirmed T0 parent plants, 31 plants had BSMV symptoms (Fig 4.2), and presence of BSMV virus was also confirmed by RT-PCR (Fig 4.1). BSMV+ T1 plants were distinguished from BSMV- T1 plants by yellowish color of their leaves (Fig 4.2). The yellowish color of leaves in BSMV+ plants was fully correlated with RT-PCR confirmation. Therefore, it was possible to segregate BSMV+ T1 plants from BSMV- plants just by their morphological characteristics.

In our study, the transmission rate of BSMV to next generation through seeds was found to be 52.49% of the total offspring from the BSMV-confirmed T0 plants. To study the impact of BSMV in the T1 generation, we collected the data for total number of tillers, total number of spikes and total number of seeds per plant.


**Figure 4.2.** Difference in color of leaves of T1 NIL-s wheat plants with and without BSMV.

There was no significant difference in number of tillers and number of spikes per plant between BSMV+ and the BSMV- offspring; however, there was a significant difference between total numbers of seeds between the two groups. The BSMV+ T1 plants had significantly lower number of seeds per plant on average compared to the BSMV- T1 plants (Fig 4.3). This means that BSMV impacts in the quantity of harvested grains in wheat. Therefore, caution should be taken with proper controls when BSMV-

based gene induction systems are used to study the function of any genes to balance the effect of BSMV infection itself in morphological characteristics.

To evaluate the overexpression of *WFhb1-1* gene in the T1 plants, three random BSMV+ and BSMV- plants were, respectively, selected and their RNA samples were analyzed by RT-qPCR. *WFhb1-1* gene expression was found ~2.5 fold higher in the BSMV+ plants compared to the BSMV- plants in the T1 generation.

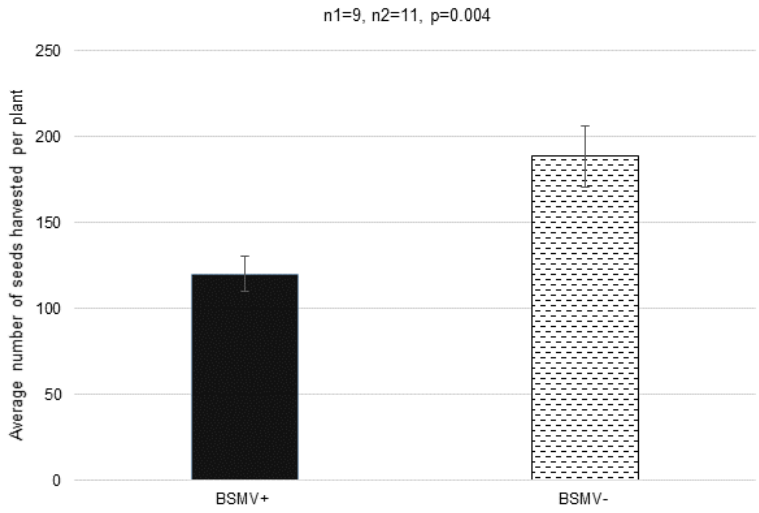


**Figure 4.3.** Comparison of number of seeds between BSMV+ and BSMV- T1 NIL-S wheat plants.

### 4.3.3 *F. graminearum* inoculation of T1 plants (BSMV:W and BSMV:C and BSMV:N)

Twenty spikes each of the three experimental groups of the T1 plants (BSMV:W, BSMV:C and BSMV:N) were inoculated with either *F. graminearum* conidia or sterile water. Here, BSMV:W-T1 means the BSMV+ T1 plants from the BSMV+ T0 plants. Similarly, BSMV:C-T1 means the BSMV- T1 plants from the BSMV+ T0 plants. Whereas, BSMV:N-T1 are the BSMV- T1 plants from the FES inoculated T0 plants.

Fusarium damaged rachides, FDR (%) and Fusarium damaged kernels, FDK (%) data was collected for three different treatments. FDR data for three treatments were collected at four different time points, 7 dpfi, 14 dpfi, 21 dpfi and 28 dpfi. The difference in FDR and FDK between BSMV:W-T1, BSMV:C-T1 and BSMV:N-T1 was evaluated and statistically analyzed using single factor ANOVA and two-tailed t-test.

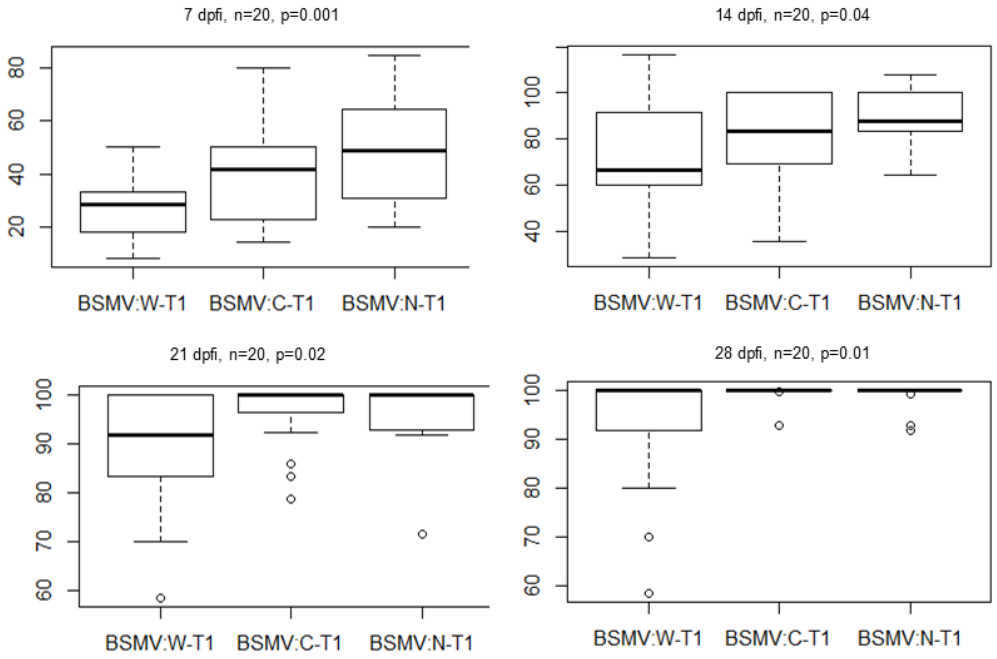


**Figure 4.4.** FDR (%) in T1 generation of plants after 7 dpfi, 14 dpfi, 21 dpfi and 28 dpfi respectively. BSMV:W-T1 is BSMV+ T1 generation from BSMV+ T0 generation. BSMV:C-T1 is BSMV- T1 generation from BSMV+ T0 generation. BSMV:N-T1 is BSMV- T1 generation from BSMV- T0 generation.

We found a significant difference in FDR between BSMV+ and BSMV- T1 plants for all four time points of analysis (Fig 4.4). This means that BSMV+ plants in T1 generation could confer resistance against FHB due to overexpression *WFhb1-1* gene,

which was passed down in these T1 plants through seeds from T0 generation. This difference was not observed in water inoculated plants.

We also analyzed FDK data between the three treatment groups; however, there was no significant difference in FDK between BSMV+ and BSMV- T1 plants. This observation might have been impacted at least partially because of difference in total number of seeds harvested between BSMV+ and BSMV- T1 plants. In the T1 generation, the level *WFhb1-1* overexpression and reduction in FHB symptoms was low (~2.5 fold) compared to the T0 generation (~30 fold as explained in Chapter 2). Nevertheless, we could verify that BSMV-induced gene overexpression could be passed down to next generation of offspring through seeds, as we could confirm the transmission of *WFhb1-1* overexpression in T1 offspring when BSMV induced VOX system was used in T0 generation.

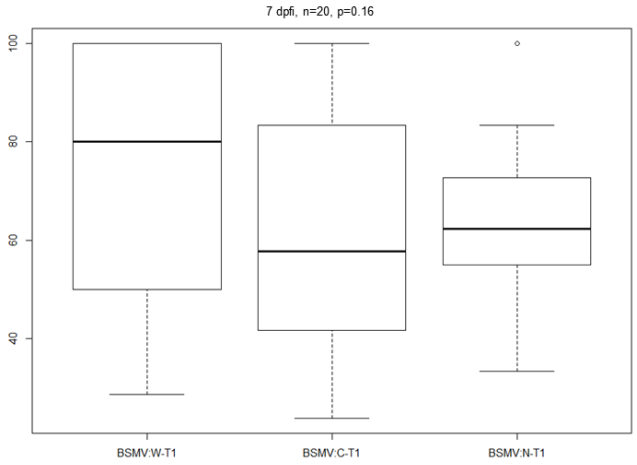


**Figure 4.5.** Fusarium damaged kernels (FDK%) between three treatment groups of T1 generation. BSMV:W-T1 is BSMV+ T1 generation from BSMV+ T0 generation. BSMV:C-T1 is BSMV- T1 generation from BSMV+ T0 generation. BSMV:N-T1 is BSMV- T1 generation from BSMV- T0 generation.

## 4.4 Discussion

BSMV-induced VIGS has been used for over a decade now to silence genes in monocot plants (Holzberg et al., 2002). However, reports on BSMV-induced VOX are very few. In this study, we overexpressed *WFhb1-1* gene using BSMV virus as a vector, and we also found the evidence of transmission of BSMV induced overexpression in next generation of wheat plants through seeds. First reports on overexpression of GFP gene using BSMV vector found a patchy overexpression of GFP protein in leaves that was not expressed uniformly (Haupt et al., 2001; Lawrence and Jackson, 2001). BSMV-based system has been improved greatly since then for the overexpression of larger sized proteins (Cheuk and Houde, 2018); however, the cargo capacity and instability of cloned gene in BSMV vector still remains as a bottleneck for the adaptation of the system widely.

It is known for a long time that BSMV can be transmitted through seeds (McKinney and Greeley, 1965). However, there are no reports if the BSMV-induced VOX can be transmitted through seeds to next generation of the treated plants. This is the first study so far in our knowledge to demonstrate the transmission of BSMV-induced VOX of a small sized gene *WFhb1-1* (384 bp) to T1 generation when the overexpression system is applied in T0 generation. However, the level of overexpression of *WFhb1-1* was lower in the T1 generation compared to T0 generation (~30 fold in T0 vs ~2.5 fold in T1). This suggests that the stability of foreign gene insert in overexpression vector during BSMV viral replication is a real challenge even if the overexpressed protein size is relatively smaller like WFhb1-1. Codon optimization for the virus might be one strategy

to make the construct more stable for multi-generational overexpression of a gene by this technique.

Similarly, we report a ~50% transmission rate of BSMV to next generation of plants from BSMV+ confirmed parents. The possibility of transfer of virus induced overexpression in new generation of plants with the transmission rate of ~50% in offspring opens wide array of possibilities in gene overexpression studies and gene editing. With this, there is a greater scope for the functional assays of genes in crop plants with larger sample sizes, when offspring with gene overexpression can be harvested from few inoculated parents.

Moreover, CRISPR/Cas based gene editing system in monocot plants has limitations because of tissue culture-based transformation process, which is more labor and cost intensive, less efficient, and stable integration of foreign gene into the genome of edited plants. Therefore, if BSMV based gene expression of CRISPR/Cas systems can be adapted for the crop plants, the expression of the *Cas9 or* any other *Cas*-gene can be eliminated in future generations by screening BSMV free offspring which have the expected gene edited. This can open possibility of gene editing in crop plants without a stable integration of foreign gene, which will avoid a lot of negative attention and regulatory hurdles related to transgenic organisms. Similarly, BSMV based system is more cost and labor efficient compared to the classical *Agrobacterium* or biolistic mediated methods of transformations and can be adapted widely by many laboratories and breeding facilities. Therefore, our study will lay a foundation for further studies to open up possibilities for broader application of BSMV virus-based gene-overexpression system (VOX) in gene overexpression and gene editing.

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## Chapter 5

### Overall discussion and future perspectives

In summary, we revealed the sequence of *WFhb1-1* gene and confirmed it as a functional gene associated with *Qfhb1*. This gene confers FHB resistance by its antifungal property, and it is a potential membrane protein. We also confirmed expression of WFhb1-1 protein in wheat and expressed this protein in yeast system using *Pichia pastoris*. We optimized BSMV virus-mediated overexpression of a gene by using 3 plasmid BSMV system to overexpress *WFhb1-1* gene in wheat. We could achieve up to 30 folds of overexpression of *WFhb1-1* gene, when the viral vector was applied on leaves of 10 days old wheat plants. We also report a ~50% transmission rate of BSMV-virus (induced overexpression) to next generation of the treated plants through seeds. As far as we know, this is the first report of transmission of BSMV virus-induced overexpression of a gene in next generation through seeds. Additionally, in this study we propose a model of DON biosynthesis down-regulation in *F. graminearum* by the infection of Fusarium graminearum virus 1 (FgV1) infection.

It has been almost two decades since *Qfhb1* has been first identified and revealed as the most reliable resistance source against FHB. Tremendous efforts and resources have been put in this research area since then. Although there has been some progress in understanding the function of *Qfhb1*, and some candidate genes have also been reported associating with this QTL by many research groups, there is no exhaustive evidence to conclude any reported candidate gene as a sole source of FHB resistance associated with *Qfhb1*. Some candidate genes have been found functionally associated with *Qfhb1* but could not be located physically within the QTL region that has been sequenced, whereas

other candidate genes are located within the QTL physically but could not be confirmed functionally. The *WFhb1-1* gene has been found to be functionally associated with *Qfhb1* but could not be located inside the sequenced contigs that are believed to harbor *Qfhb1* (Paudel et al., 2017; Zhuang et al., 2013). Although *WFhb1-1* lies within the first reported markers, *Xgwm533* and *Xgwm493*, it lies off the region included by UMN10 markers that were developed later. Similarly, no *TaUGT* gene could be physically located in the *Qfhb1* region when a contig harboring *Qfhb1* was sequenced by Schweiger et al. (2016). On the other hand, the reported candidate genes that are physically located in the *Qfhb1* region, such as *TaPFT* and *TaGDSL*, are also reported to be present in wheat genotypes that lack *Qfhb1* (Su et al., 2019; Li et al., 2019). Moreover, Li et al. (2019) reported that *TaPFT* gene is outside *Qfhb1* in their mapping study. *TaHRC* as a candidate gene that confers susceptibility when present in wheat genotypes, as reported by Su et al. (2019), contradicts the findings by Li et al. (2019) as the former claim a deletion mutation confers resistance whereas the later claim frameshift mutation that encodes new proteins to confer resistance.

Hence, the mechanism of resistance associated with *Qfhb1* still remains a complex and poorly understood area requiring further exploration. Multiple gene interactions in resistant genotypes that might include genes within the *Qfhb1* region along with genes that lie beyond *Qfhb1* cannot be eliminated as a possible scenario in disease resistance. It is likely that a regulatory gene or a component within the *Qfhb1* region might regulate other resistance genes outside of the region, thus resulting the differential degree of disease severity. Therefore, the possibility of interaction between multiple

genes/regulatory elements within and beyond the *Qfhb1* region, including but not limited to the reported *Qfhb1* candidate genes, should be considered in future studies.

Here, we propose a two gene model of resistance associated with *Qfhb1* (Fig 5.1). In our two gene model of resistance, pathogen might interact with gene 1, and its expression regulation might work as a switch to control the expression of downstream gene (gene 2). Gene two is a functional gene that works against pathogen, which confers resistance. Based on the published information, gene 1 might be *TaHRC* (or *TaHis*), and regulation of expression of this gene might control downstream functional genes like *WFhb1-1*.

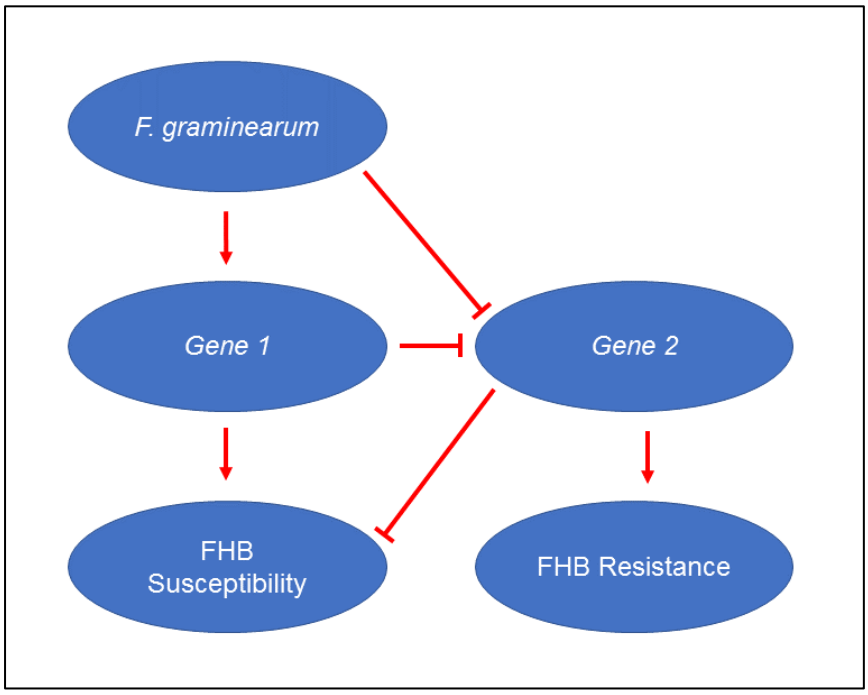


**Figure 5.1.** Two gene model of *Qfhb1* mediated resistance against FHB.

In many communications, there seems to be a confusion about *Qfhb1* as a candidate gene than as a QTL marker. As the presence of *Qfhb1* accounts for FHB

resistance, many breeders and biologists like to think that there should be a single gene present in *Qfhb1+* wheat lines and absent in *Qfhb1-* lines, which confers the FHB resistance. Although this is reasonable hypothesis, many studies with the objectives to identify a candidate gene associated with *Qfhb1* indicate that *Qfhb1* is rather a big and complex region having many potential candidate genes along with possible regulatory components. The genes or any other potential regulatory components within *Qfhb1* might also regulate other genes inside or outside of this QTL. Therefore, we should be forbearing and open minded for different possibilities towards our further endeavors of understanding the overall role of *Qfhb1* in FHB resistance.

FHB remains one of the most damaging fungal disease of small grain crops. Therefore, alternative management approaches like the use of biocontrol agents have a potential if biocontrol agents could be developed for broader application. Fusarium graminearum virus 1 (FgV1) has such a potential because of its ability to make *F. graminearum* hypovirulent. FgV1 can suppress *dcl2*, *ago1,* and/or *Dicer2* in *F. graminearum* to suppress the siRNA mediated anti-viral response in the host fungus, which is associated with hypovirulence due to FgV1 infection and lower level of DON accumulation in infected wheat seeds by the fungus (Yu et al., 2018). However, the mechanism on how FgV1 decreases the level of DON accumulation is unknown. In our study we found that FgV1-SD4 strain when infecting *F. graminearum,* can eliminate DON accumulation in infected wheat seeds, and we propose that the virus eliminates this mycotoxin through regulation of siRNA named as *fg*siR34. We propose a pathway of DON downregulation by FgV1 by downregulation of *dcl2*, which downregulates siRNA

*fg*siR34. Downregulation of *fg*siR34 suppresses the expression of *Tri* genes, which ultimately results in interruption of DON biosynthesis.

If an extracellular method of transfection by FgV1 could be developed, it will open a possibility for application of this virus as a biocontrol agent. However, until new tools of management are developed, the resistant wheat cultivars that are widely used only have modest level of resistance, and fungicidal control of the disease has its own limitations. Identification and cloning of candidate genes associated with limited available resistant QTLs has not helped much either in the development of more resistant cultivars yet. Therefore, combinatorial approach of all the available FHB management techniques remains the best strategy to cope the epidemics of FHB and economic challenges associated with it.

## 5.1 Reference

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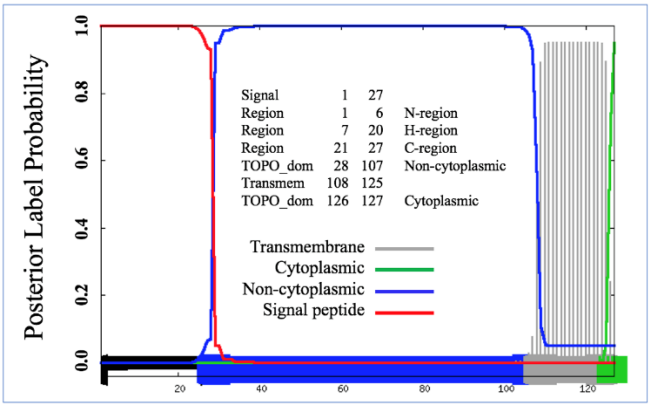
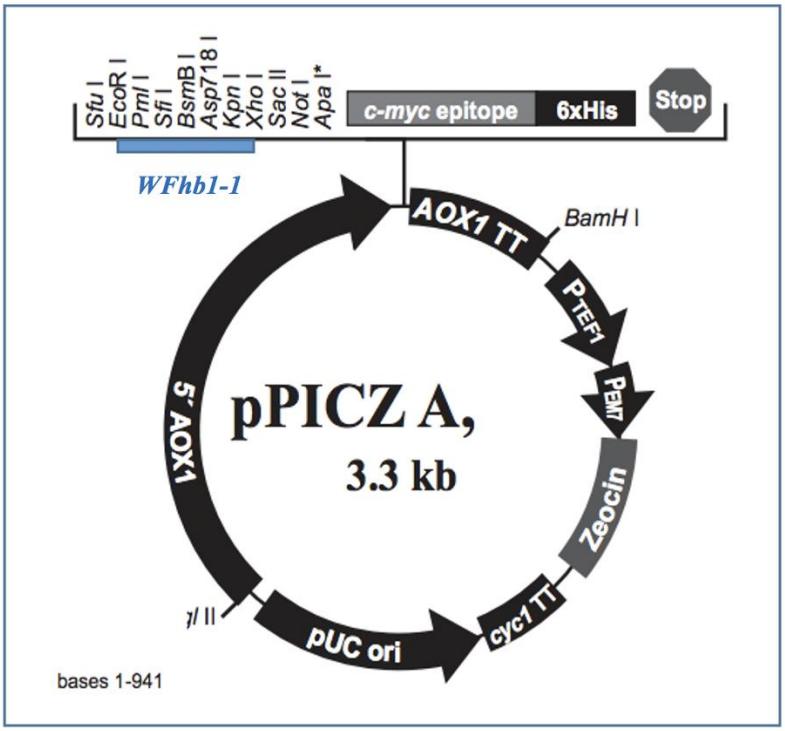
# Appendix 1

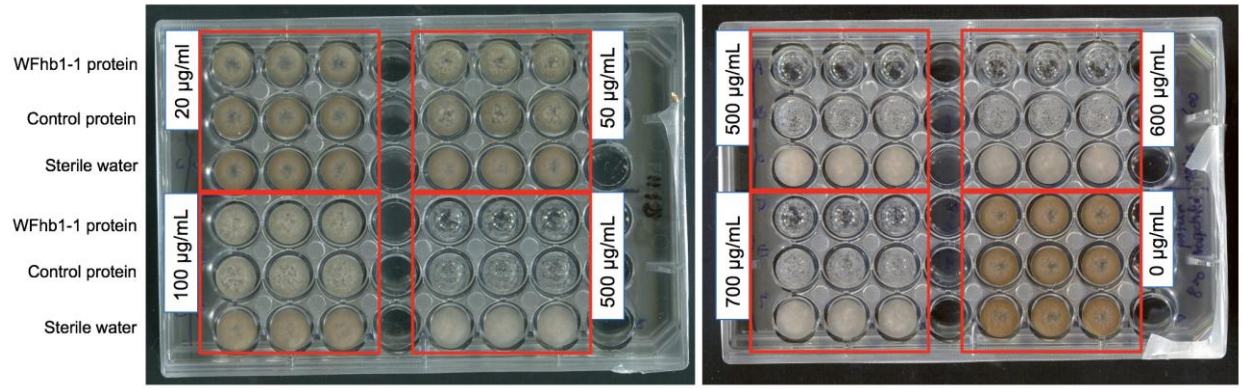
## Supplementary files for Chapter 2



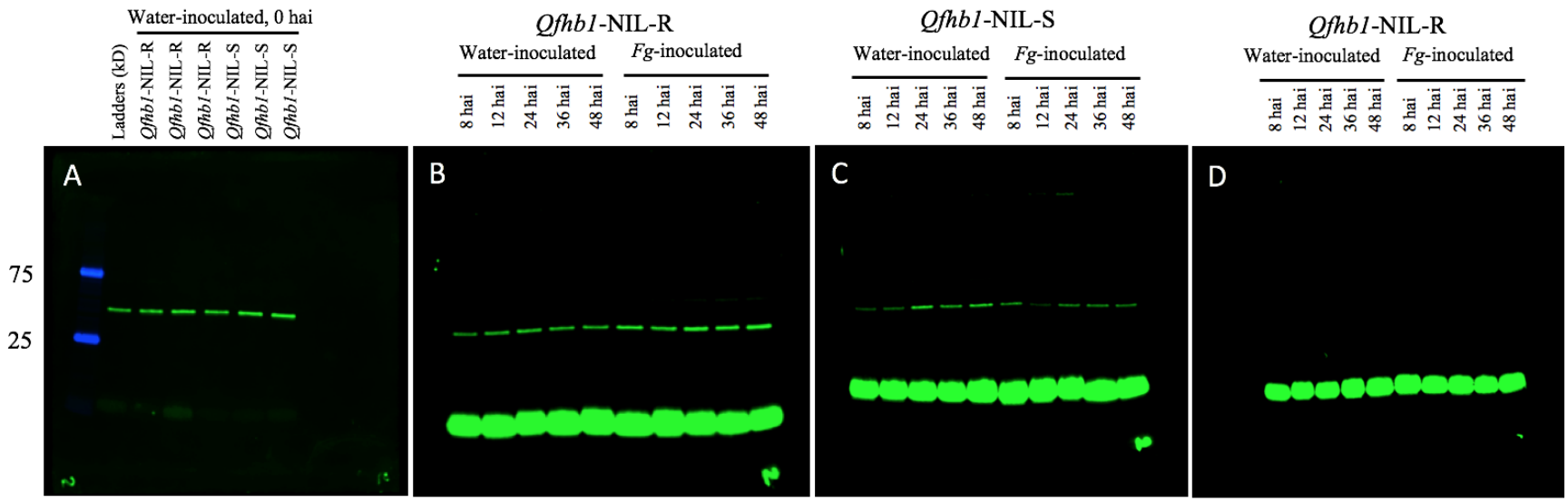

**Supplementary Figure S2.1** A graphic illustrating the properties of WFhb1-1 protein predicted by Phobius.



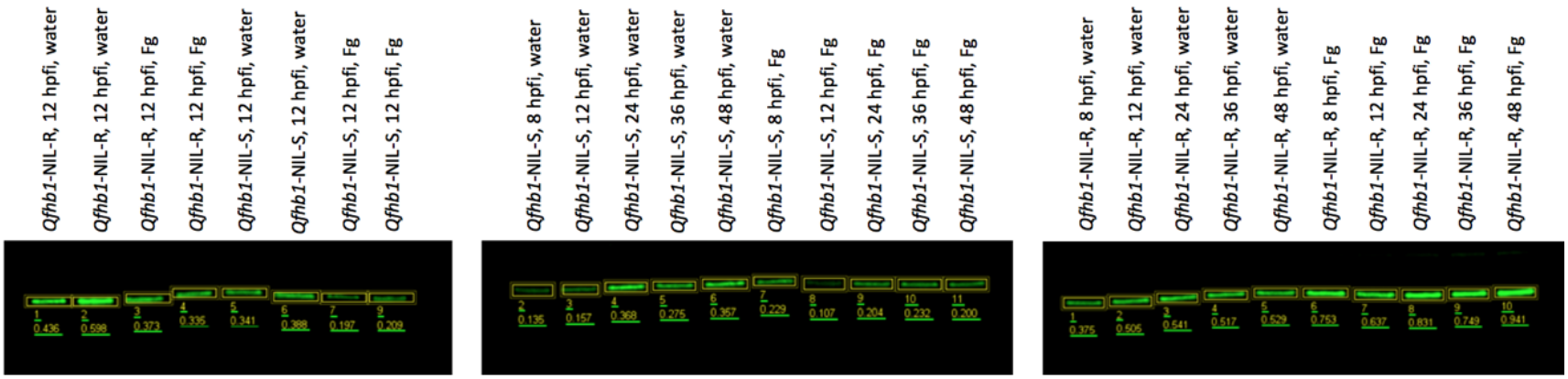

**Supplementary Figure S2.2** An illustration of the *WFhb1-1* cloning position in *pPICZA* plasmid.



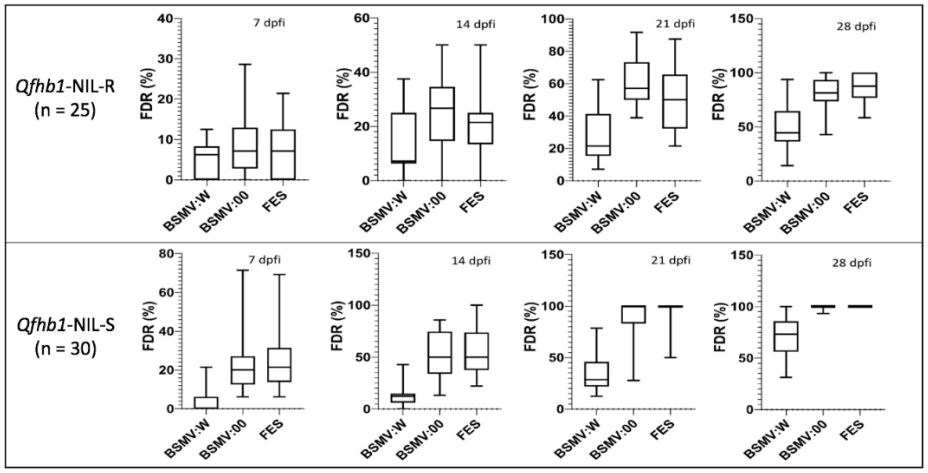

**Supplementary Figure S2.3** Photos showing growth of 1000 conidia of *Fusarium graminearum* in 100 µL potato dextrose broth supplemented with, 0, 20, 50, 100, 500, 600 or 700 µg/mL total protein isolated from *WFhb1-1*-expression *Pichia pastoris* X33:T, wildtype X33:00 or sterile water. The photo was taken two weeks after the culture started.



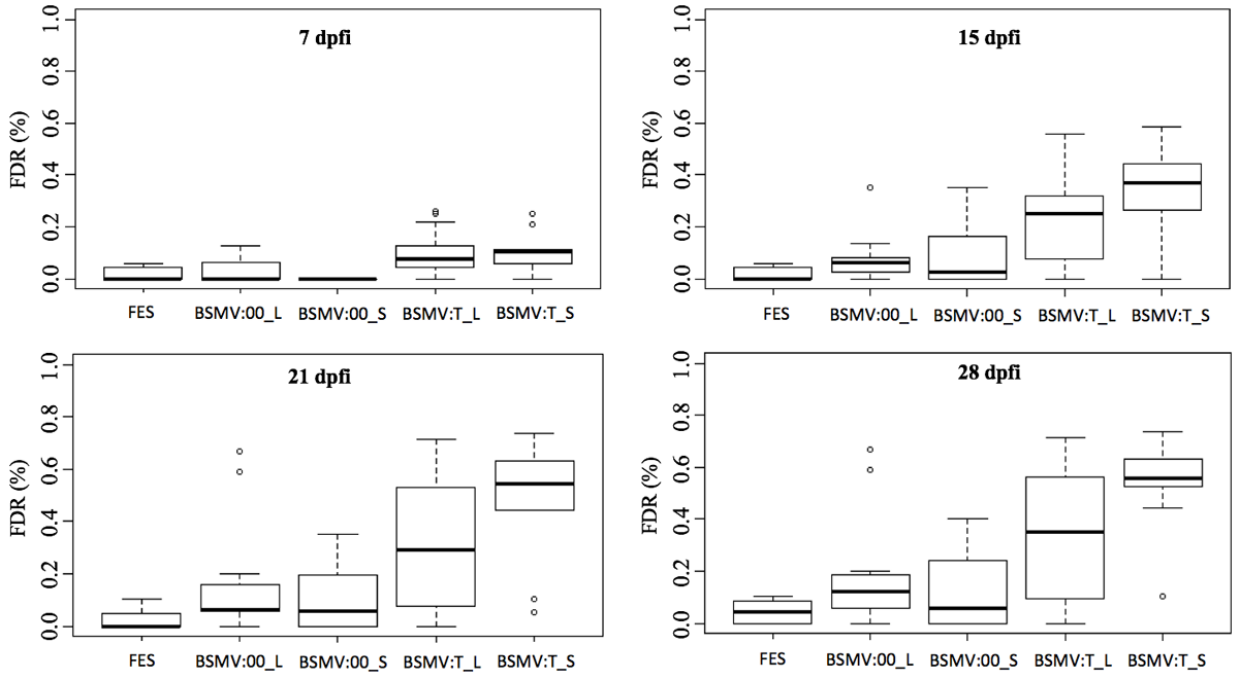

**Supplementary Figures S2.4** The original photos used to prepare panels **A, B** and **C** in Figures 3, respectively. **D:** a gel loaded with the same samples as in **B** but was not hybridized with anti- WFhb1-1 antibody. *Fg*: *Fusarium graminearum*. hai: hours after inoculation.



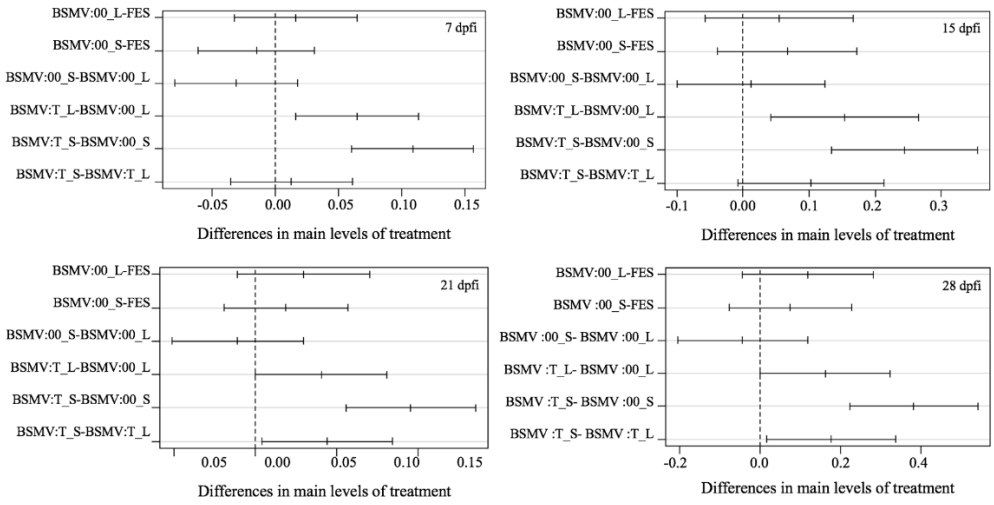

**Supplementary Figure S2.5** Photos of representative images of digitizing fluorescence signal strength for each band on the Western Blots of WFhb1-1 protein probed with anti- WFhb1-1 antibody PA-2.




**Supplementary Figure S2.6** Graphics showing mean *Fusarium* damaged rachis rate (FDR)) of *Qfhb1*-NIL-S and *Qfhb1*-NIL-R of the *WFhb1-1*-overexpression plants (BSMV:W) and controls (BSMV:00 and FES) in 7, 14, 21 and 28 days post FHB-inoculation (dpfi).

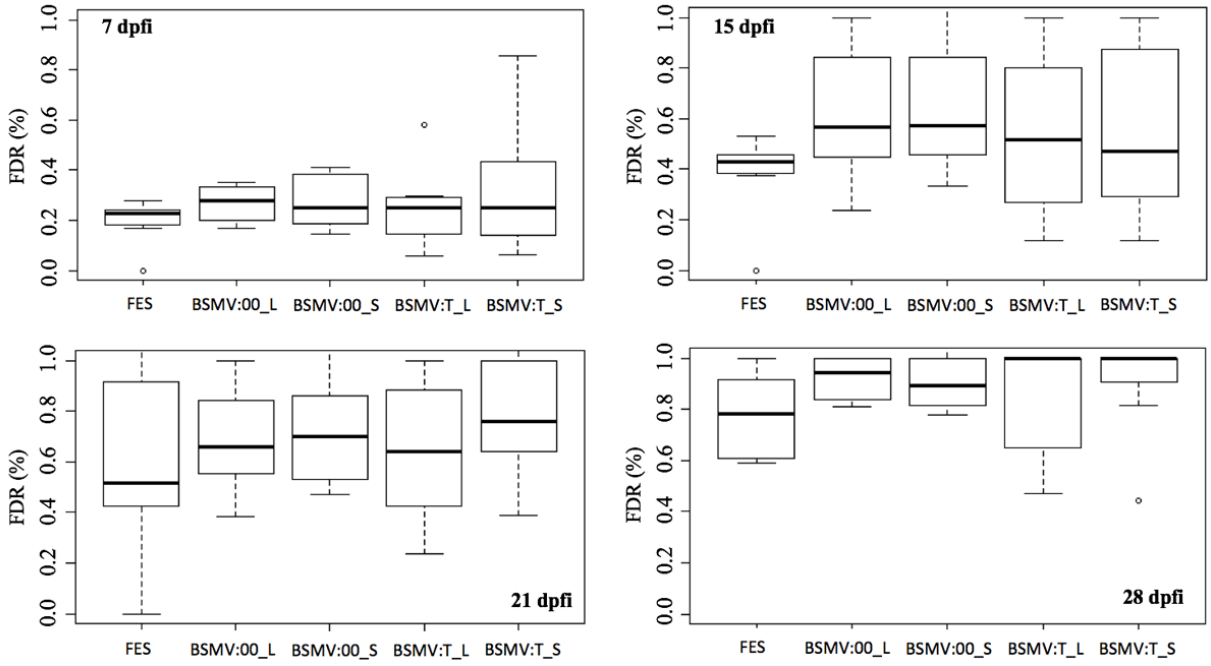



**Supplementary Figure S2.7** Graphics showing mean FHB-damaged rachis rates (FDR) of Sumai 3 plants inoculated first with the inoculation buffer (FES), the empty vector control (BSMV:00) or *WFhb1-1*-silencing vector (BSMV:T) and then with *Fusarium graminearum* in 7, 15, 21 and 28 days post *Fusarium* inoculation (dpfi). _L: leaf viral inoculation; _S: spike viral inoculation.

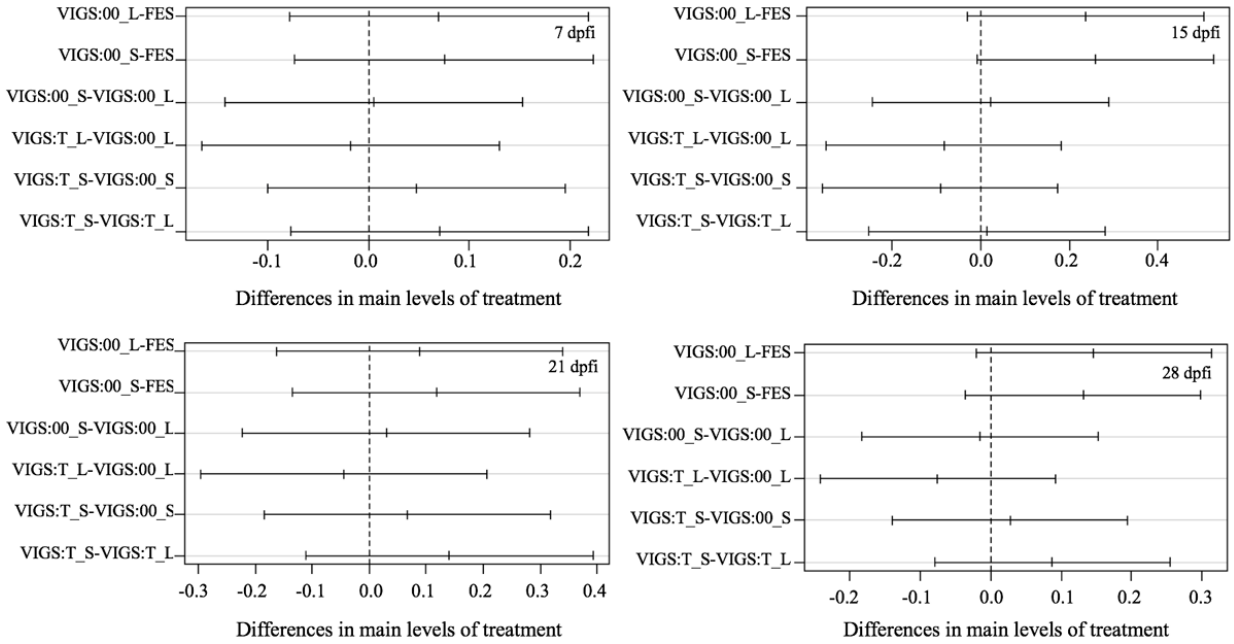



**Supplementary Figure S2.8** Graphics showing the results of Turkey multiple comparisons of means between VIGS treatments at 95% family-wise confidence level for Sumai 3 in 7, 15, 21- and 28-days post *Fusarium* inoculation (dpfi).


**Supplementary Figure S2.9** Photos show typical phenotypes of Sumai 3 spikes inoculated first with *WFhb1-1*-silencing BSMV:T and 15 days later with *Fusarium graminearum*. dpfi: days post *Fusarium* inoculation.




**Supplementary Figure S2.10** Graphics showing mean FHB diseased rates (FDR) of Y1193-06 plants inoculated first with the inoculation buffer (FES), the empty vector control (BSMV:00) or *WFhb1-1*-silencing vector (BSMV:T) and then with *Fusarium graminearum* in 7, 15, 21 and 28 days post *Fusarium* inoculation (dpfi). _L: leaf viral inoculation; _S: spike viral inoculation.




**Supplementary Figure S2.11** Graphics showing the results of Turkey multiple comparisons of means between VIGS treatments at 95% family-wise confidence level for Y1193-06 in 7, 15, 21 and 28 days post *Fusarium* inoculation (dpfi).

**Supplementary Document S2.1**: Formulas for reagents and the culture media used in this study.

**Low salt LB (Luria-Bertani) Medium:**

1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, pH 7.5 adjusted with 1 N NaOH.

For LB Agar plates: 15 g/liter agar added before autoclaving.

**YPD or YEPD (Yeast Extract Peptone Dextrose Medium):**

1% yeast extract, 2% peptone, 2% dextrose (glucose)

**10X YNB (Yeast Nitrogen Base):**

13.4% Yeast Nitrogen Base with Ammonium Sulfate without amino acids

**500X B (0.02% Biotin):**

Dissolve 20 mg biotin in 100 ml of water and filter sterilize. Store at 4°C.The shelf life of this solution is approximately one year.

**10X M (5% Methanol):**

Mix 5 ml of methanol with 95 ml of water. Filter sterilize and store at 4°C. The shelf life of this solution is approximately two months.

**MGY (Minimal Glycerol Medium):**

1.34% YNB, 1% glycerol, $4 \times 10^{-5}$% biotin

Combine aseptically 800 ml autoclaved water with 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X GY. Store at $4^{o}$ C. The self-life of this solution is approximately two months.

**MM (Minimum Methanol):**

1.34% YNB, 4 × 10–5% biotin, 0.5% methanol.

For liquid medium, autoclave 800 ml of water for 20 minutes on liquid cycle. Cool autoclaved water to 60°C and add: 100 ml of 10X YNB, 2 ml of 500X B, 100 ml of 10X M.

MM stores well for several months at 4°C.

**Breaking buffer:**

50 mM sodium phosphate (pH 7.4), 1 mM protease inhibitors, 1 mM EDTA, 5% glycerol.

**2X Laemmli buffer:**

0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.004% Bromophenol blue, 10% beta-mercaptoethanol.

**TBST (Tris-buffered Saline with Tween 20) buffer:**

20 mM Tris (pH 7.5), 150 mM Nacl, 0.1% Tween 20

**Blocking buffer:**

5% dry milk powder in TBST buffer

**FES buffer:**

Sodium-pyrophosphate [1%, wt/vol], macaloid [1%, wt/vol], celite [1%, wt/vol], 0.5 M glycine, and 0.3 M K2HPO4, pH 8.5, with phosphoric acid.

**CMC medium:**

Dissolve 15 g of carboxymethyl cellulose sodium salt (Sigma-Aldrich), 1 g NH4NO3, 1 g KH2PO4, 0.5 g MgSO4.7H2O, and 1 g yeast extract in 1 L water. Heat and stir to dissolve. Aliquot 100 ml apiece into 250-ml flasks, autoclave for 20 min

**Supplementary Table S2.1** Measurement and statistical analysis of fluorescence strength from the Western Blots of WFhb1-1 protein isolated from *Fusarium graminearum*- or water-inoculated spikelets of *Qfhb1*-nesr-isogenic line pair NIL-R (carrying *Qfhb1*) and NIL-S (not carrying *Qfhb1*) detected with anti- WFhb1-1 antibody PA-2. **(**hpfi: hours post *Fusarium* inoculation)

| NIL-R | Water-inoculated | | | | | | *Fusarium graminearum*-inoculated | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hpfi | 8 hpfi | 12 hpfi | 24 hpfi | 36 hpfi | 48 hpfi | 0 hpfi | 8 hpfi | 12 hpfi | 24 hpfi | 36 hpfi | 48 hpfi |
| Rep 1 | 0.292 | 0.375 | 0.505 | 0.541 | 0.517 | 0.529 | 0.292 | 0.753 | 0.637 | 0.831 | 0.749 | 0.941 |
| Rep 2 | 0.359 | 0.437 | 0.436 | 0.414 | 0.587 | 0.807 | 0.359 | 0.37 | 0.373 | 0.651 | 0.447 | 0.731 |
| Rep 3 | 0.457 | 0.414 | 0.598 | 0.572 | 0.515 | 0.580 | 0.457 | 0.504 | 0.335 | 0.203 | 0.692 | 0.926 |
| Rep 4 | | | 0.235 | | | | | | 0.285 | | | |
| Rep 5 | | | 0.444 | | | | | | 0.261 | | | |
| Average | 0.369 | 0.409 | 0.444 | 0.509 | 0.540 | 0.660 | 0.369 | 0.542 | 0.378 | 0.562 | 0.629 | 0.866 |
| SD | 0.083 | 0.031 | 0.133 | 0.084 | 0.041 | 0.184 | 0.083 | 0.194 | 0.151 | 0.323 | 0.160 | 0.117 |
| SE | 0.048 | 0.018 | 0.060 | 0.048 | 0.024 | 0.106 | 0.048 | 0.112 | 0.068 | 0.187 | 0.093 | 0.068 |
| T-test | 0.171 | | | | | | | 0.305 | 0.489 | 0.789 | 0.401 | 0.177 |
| Normalized | 0.369 | | | | | | | | | | | |
| Fold change | 1.000 | 1.016 | 1.369 | 1.466 | 1.401 | 1.434 | 1.000 | 2.041 | 1.726 | 2.252 | 2.030 | 2.550 |
| | | 1.184 | 1.182 | 1.122 | 1.591 | 2.358 | | 1.003 | 1.011 | 1.764 | 1.211 | 1.981 |
| | | 1.122 | 1.621 | 1.550 | 1.396 | 1.572 | | 1.366 | -1.101 | -1.818 | 1.875 | 2.509 |
| | | | -1.570 | | | | | | -1.295 | | | |
| | | | 1.203 | | | | | | -1.414 | | | |
| Ave | 0.000 | 1.107 | 0.761 | 1.379 | 1.463 | 1.788 | 0.000 | 1.470 | -0.215 | 0.733 | 1.706 | 2.347 |
| SD | 0.000 | 0.085 | 1.315 | 0.227 | 0.111 | 0.498 | 0.000 | 0.527 | 1.471 | 2.222 | 0.435 | 0.317 |
| SE | 0.000 | 0.049 | 0.588 | 0.131 | 0.064 | 0.288 | 0.000 | 0.304 | 0.658 | 1.283 | 0.251 | 0.183 |

| NIL-S | Water-inoculated | | | | | | *Fusarium graminearum*-inoculated | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 hpfi | 8 hpfi | 12 hpfi | 24 hpfi | 36 hpfi | 48 hpfi | 0 hpfi | 8 hpfi | 12 hpfi | 24 hpfi | 36 hpfi | 48 hpfi |
| Rep 1 | 0.384 | 0.135 | 0.157 | 0.368 | 0.275 | 0.357 | 0.384 | 0.229 | 0.107 | 0.204 | 0.232 | 0.200 |
| Rep 2 | 0.521 | 0.324 | 0.341 | 0.109 | 0.479 | 0.432 | 0.521 | 0.360 | 0.197 | 0.231 | 0.217 | 0.617 |
| Rep 3 | 0.609 | 0.459 | 0.388 | 0.477 | 0.438 | 0.548 | 0.609 | 0.441 | 0.209 | 0.418 | 0.557 | 0.510 |
| Rep 4 | | | 0.434 | | | | | | 0.168 | | | |
| Rep 5 | | | 0.402 | | | | | | 0.123 | | | |
| Average | 0.505 | 0.306 | 0.344 | 0.318 | 0.397 | 0.446 | 0.505 | 0.343 | 0.161 | 0.284 | 0.335 | 0.442 |
| SD | 0.113 | 0.163 | 0.110 | 0.189 | 0.108 | 0.096 | 0.113 | 0.107 | 0.045 | 0.117 | 0.192 | 0.217 |
| SE | 0.065 | 0.094 | 0.049 | 0.109 | 0.062 | 0.056 | 0.065 | 0.062 | 0.020 | 0.067 | 0.111 | 0.125 |
| T-test | 0.171 | | | | | | | 0.757 | 0.009 | 0.235 | 0.112 | 0.982 |
| Normalized | 0.505 | | | | | | | | | | | |
| Fold change | 1.000 | -3.741 | -3.217 | -1.371 | -1.835 | -1.414 | 1.000 | -2.205 | -4.720 | -2.475 | -2.177 | -2.525 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | -1.559 | -1.481 | -1.481 | -4.630 | -1.053 | | -1.403 | -2.563 | -2.186 | -2.327 | 1.222 |
| | | -1.100 | -1.302 | -1.058 | -1.152 | 1.086 | | -1.145 | -2.416 | -1.208 | 1.103 | 1.010 |
| | | | -1.164 | | | | | | -3.006 | | | |
| | | | -1.256 | | | | | | -4.106 | | | |
| Ave | 0.000 | -2.133 | -1.684 | -2.353 | -1.347 | -0.499 | 0.000 | -1.584 | -3.362 | -1.957 | -1.134 | -0.098 |
| SD | 0.000 | 1.411 | 0.865 | 1.978 | 0.426 | 1.378 | 0.000 | 0.553 | 1.007 | 0.664 | 1.938 | 2.105 |
| SE | 0.000 | 0.815 | 0.387 | 1.142 | 0.246 | 0.796 | 0.000 | 0.319 | 0.450 | 0.383 | 1.119 | 1.215 |

**Supplementary Table S2.2** Measurements and statistical analysis of deoxynivalenol in the kernels harvested from *Fusarium*-inoculated spikes of *Qfhhb1* near-isogenic lines NIL-R (carrying *Qfhb1*) and NIL-S (not-carrying *Qfhb1*) inoculated with *WFhb1-1*-overexpressing BSMV:W, wildtype BSMV:00 or viral inoculation buffer FES.

| NIL-S | BSMV:W | BSMV:00 | FES | NIL-R | BSMV:W | BSMV:00 | FES |
|---|---|---|---|---|---|---|---|
| **Exp1** | 1.215774 | 14.07535 | 6.161836 | **Exp1** | 0.655526 | 0.300615 | 0.934874 |
| | 3.126315 | 41.69496 | 2.220297 | | 2.065056 | 0.190049 | 0.350029 |
| | 11.98365 | 11.29983 | 8.33749 | | 0 | 0.199467 | 1.37028 |
| | 5.028774 | 8.09783 | 13.21569 | | 0.31215 | 0.096184 | 0.087819 |
| | 7.571769 | 0.587178 | 7.344306 | | 3.774041 | 16.96128 | 6.746974 |
| | 2.939713 | 9.612612 | 1.192799 | | 0 | 3.596712 | 2.419767 |
| | 5.81554 | 32.33572 | 6.273164 | | 0 | 0.070522 | 0.056148 |
| | 0.421495 | 14.13058 | 8.351239 | | 0.079412 | 1.968261 | 0.053421 |
| | 6.26241 | 1.524113 | 16.37352 | | 17.64183 | 7.279542 | 0.53508 |
| | 11.91572 | 10.81543 | 18.52021 | | 0 | 0 | 0.484565 |
| | 15.83737 | 4.594647 | 3.13002 | | 8.458574 | 1.65449 | 2.723375 |
| | 0.327753 | 40.37845 | 2.988545 | | 0.072951 | 2.848856 | 2.110902 |
| | 0 | 13.50766 | 15.24438 | | 0 | 0 | 0 |
| | 5.520804 | 8.706757 | 4.964507 | | 0 | 0 | 0 |
| | 5.605407 | 46.2993 | 7.833133 | | 0 | 6.770157 | 0 |
| | 0.426047 | 6.076089 | 4.562935 | | | | |
| | 0 | 0.741639 | 6.244772 | | | | |
| | 0.41127 | 0 | 12.87445 | | | | |
| | 0.392817 | 0.586586 | 10.42028 | | | | |
| | 2.249001 | 8.936259 | 1.290686 | | | | |
| **Exp2** | 0.809095 | 7.898423 | 14.65127 | **Exp2** | 2.435716 | 1.362592 | 0.170926 |
| | 1.742926 | 6.74183 | 27.95262 | | 0 | 3.882156 | 0.772399 |
| | 3.155238 | 4.035355 | 2.723276 | | 0 | 7.720536 | 0.229194 |
| | 3.055736 | 2.311778 | 32.81303 | | 0.605462 | 1.359589 | 0 |
| | 1.239757 | 13.06527 | 0.320438 | | 0.211784 | 0 | 1.946175 |
| | 0 | 0.739216 | 31.27817 | | 0.248218 | 1.64968 | 0.86921 |
| | 0 | 10.08995 | 6.424545 | | 0.893988 | 0.143852 | 0.790312 |
| | 0 | 0 | 0 | | 1.662322 | 0.25483 | 8.70344 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 0.746012 | 0 | 0 | 12.57758 | 2.455242 |
| | 0 | 0 | 0 | 0.025349 | 2.783813 | 1.121645 |
| **Ave** | 3.235146 | 10.65429 | 9.123587 | 1.565695 | 2.94683 | 1.397271 |
| **SD** | 4.042492 | 12.59088 | 8.828327 | 3.821606 | 4.303079 | 2.108828 |
| **SE** | 0.738055 | 2.29877 | 1.611825 | 0.764321 | 0.860616 | 0.421766 |
| **T-test:** | | | | | | |
| **Exp1** | | 0.009181 | 0.09878 | | 0.734362 | 0.219431 |
| **Exp2** | | 0.031289 | 0.146591 | | 0.0645 | 0.345112 |
| **Combined** | | 0.003741 | 0.593976 | | 0.236058 | 0.112474 |

# Appendix 2

## Supplementary files for Chapter 3

**Supplementary file S3.1** Sequence alignment of FgV1-SD4 with FgV1-DK21 with BLAST



**BLAST ® » blastn suite » results for RID-WT05SC0R015**

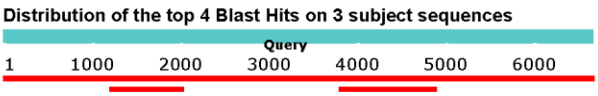
Your search is limited to records that include: Fusarium graminearum dsRNA mycovirus-1 (taxid:194397)

| | |
|---|---|
| **Job Title** | FgV1-NC_006937.2 ... |
| **RID** | WT05SC0R015 Search expires on 11-15 05:16 am |
| **Results for** | 1:lcl\|Query_65314 None(6623bp) |
| **Program** | BLASTN |
| **Database** | nt |
| **Query ID** | lcl\|Query_65314 |
| **Description** | None ... |
| **Molecule type** | dna |
| **Query Length** | 6623 |

**Descriptions**

| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Fusarium graminearum dsRNA mycovirus-1 strain DK-21, complete genome | 10739 | 10799 | 100% | 0.0 | 95.94% | AY533037.2 |
| Fusarium graminearum dsRNA mycovirus-1 clone pDK-2 sequence | 1712 | 1712 | 16% | 0.0 | 95.29% | AF443212.1 |
| Fusarium graminearum dsRNA mycovirus-1 clone pDK-1 sequence | 1310 | 1310 | 12% | 0.0 | 95.40% | AF443213.1 |

**Graphic Summary**




**Alignments**

Alignment view Pairwise CDS feature

Fusarium graminearum dsRNA mycovirus-1 strain DK-21, complete genome
Sequence ID: **AY533037.2** Length: 6621 Number of Matches: 2
Range 1: 1 to 6621

| Score | Expect | Identities | Gaps | Strand | Frame |
|---|---|---|---|---|---|
| 10739 bits(5815) | 0.0() | 6355/6624(96%) | 4/6624(0%) | Plus/Plus | |

```
Query  1    GGGGTATACTCTGAATTATTTGAATTTATTCGAGTTTTGGGGTGTAACCTGAGATGCCTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGGGTATACTCTGAATTATTTGAATTTATTCGAGTTTTGGGGTGTAACCTGAGATGCCTG  60

Query  61   ATGATCTTAATGTGAAATCCCCGGATAGCATCAAAATGCAGTTCGCACATTATTTGCTCA  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ATGATCTTAATGTGAAATCCCCGGATAGCATCAAAATGCAGTTCGCACATTATTTGCTCA  120

Query  121  ATTGGCCAACATGGCTCATTCGGATCATTGCCACTTTGTGGTTACTTAGTTTTATTCTGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ATTGGCCAACATGGCTCATTCGGATCATTGCCACTTTGTGGTTACTTAGTTTTATTCTGC  180

Query  181  CGTTGTTTTTGCTTCTCACAGTGCTACCCTTTGGGGTTTTTGCAGTTGCAATTGCATTTT  240
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  181  CGTTGTTTTTGCTTCTCACAGTGCTGCCCTTTGGGGTTTTTGCAGTTGCAATTGCATTTT  240

Query  241  TCATTCAGGGTGGCATCCTGACTTGGGCCGTTTTCCttttttttCGTCCCCTTCTTCGTCA  300
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Sbjct  241   TCATTCAGGGTGGCATCCTGACTTGGGCCGTTTTCCTTTTTTTCGTCCCTTTCTTCGTCA  300

Query  301   TTATATGCAGTAAGTTCTGGCGTCGCGCCATAGTCAAGGAGAAAGCCCTCCGCGATGCCC  360
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Sbjct  301   TTATATGCAGTAAGTTCTGGCGTCGCGCCATAGCCAAGGAGAAAGCTCTCCGCGATGCCC  360

Query  361   TGGGGCCGGCGGCTTTTCTGTGGTATGACGAGGAGAAAACCGCAGCTGGTGTCGTGTTGC  420
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Sbjct  361   TCGGGCCGGCGGCTTTTCTGTGGTATGACGAGGAGAAAACCGCAGCTGGTGTTGTGTTGC  420

Query  421   GTCCGGTCGTCATGGCCGGTTGGGATTTGATTGTCCCAGCTGCCGGTGATGCCTTGCGCT  480
              |||||||||||||||||| ||||| |||||||||||||||||| |||||||| ||||
Sbjct  421   GTCCGGTCGTCATGGCCGGCTGGGACTTGATTGTCCCAGCTGCCGGCGATGCCTTACGCT  480

Query  481   CTGCCCTGCCTGCTCAATGGCAGGCTGTCATTGGCGACTTCCCGATGTCGACAGATGAAC  540
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Sbjct  481   CTGCTCTGCCTGCTCAATGGCAGGCTGTCATTGGCGACTTCCCAATGTCGACAGATGAAC  540

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Sbjct  541   TAGCTTCTTGGTCTTGGAGTTACCTCATTGACTTTGCGGATTGGATGACTTGCGGGCCGG  600

Query  601   CAGTCGGTGTTGTTATGTTCGACATGGCTGTCCTGGCCAGGCTCTTCGCCTTGCACAGGC  660
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Sbjct  601   CAGTCGGTGTTGTCATGTTTGACATGGCTGTCCTGGCCAGGCTCTTCGCTTTGCACAGGC  660

Query  661   TGTCTTTCAGGCTCGCAAAGAAGGTGATTTTCCTTTCTGCTTTGGCCTTTTCGCTCATTT  720
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Sbjct  661   TGTCTTTCAGGTTGGCAAAGAGGGTCATTTTCCTTTCTGCTTTGGCCTTTTCGCTCATTT  720

Query  721   GGGCCGCGAGTCCTGAATTCCTTCTCGGCTGTGCCTTTGCCCTTGATCATTTCATCATGC  780
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Sbjct  721   GGGCCGCGAGTCCTGAATTCCTTCTCGGCTGTGCCTTTGCCCTTGATCATTTTATCATGC  780

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Sbjct  781   TGGTTTCGTCAGGCACTTTGGGCGAGTGGCTGCGCTGGAGGCTGACACATCTTGTGGTTG  840

Query  841   TCTGTGCTGTCTGGGCTCTGGACGTTTCGACTGTCTCTCGCAAGTACACGCCTGTTGCCT  900
              | |||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||| |
Sbjct  841   TTTGTGCTGTCTGGGCTCTGGACGTTTCGACTGTCTCTCGCAAGTACACTCCTGTTGCTT  900

Query  901   TTGGCCAGCTGGGTGGTCGCGGAAGGCATCGGAACTTTGCCGGTCTGTTCAGGGAGAAGC  960
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Sbjct  901   TTGGCCAGCTGGGTGGTCGCGGAAGGCACCGGAACTTTGCTGGCCTGTTCAGGGAGAAGC  960

Query  961   TTATGCAGGCCACAGTCTTTGTTTCTGATCTTGGCCTGCCTCACTACATCCGTGGCACCA  1020
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Sbjct  961   TAATGCAAGCTACAGTCTTTGTTTCTGATCTTGGCCTTCCCCACTACATCCGCGGCACCA  1020

Query  1021  GACCCATGACACGTCAGGGCCTTGACGAGAGCCTTGATGTGCTCCGTGAGCTTGGTTGGC  1080
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Sbjct  1021  GGCCCATGACACGTCAGGGCCTCGACGAGAGCCTTGATGTGCTCCGTGAGCTTGGTTGGC  1080

Query  1081  CTGTTAATGTTCGCACCAACGATAATGTTGCAGAGGTCACTGACAGGCTCGGGTTTAGAG  1140
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Sbjct  1081  CTGTCAATGTTCGCACCAACGATAATGTTGCAGAGGTCACTGACAGGCTCGGGTTTAGAG  1140

Query  1141  AGTGGCTGCTCTGCGGTACCGACTGGGAGCAAGGCATTCGCAACCTTAAGACGTATACCG  1200
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Sbjct  1141  AGTGGCTGCTCTGCGGCACCGACTGGGAGCAAGGCATTCGCAATCTCAAGACGTACACCG  1200

Query  1201  ATGAGTTGTTGGATGACCTGAGACTCCAGGCCGTTGAATTCCGACGCACTGAGGAGTATG  1260
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Sbjct  1201  ATGAGCTGTTGGATGACCTGAGACTTCATGCCGTTGAGTTCCGACGCACTGAGGAGTATG  1260

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Sbjct  1261  CCAGCCTCGACAATGAGTTGAAATCAACCTCACGCTATTTCAGGTCGCCCCGTTACGATT  1320

Query  1321  TTCCTGATTTGGAACTGGACGACGTGTGGTTTTTGGTCAAGGATACTTTTCAGCACTCGA  1380
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Sbjct  1321  TCCCTGATTTGGAACTGGACGACGTGTGGTTTTTGGTCAAGGACACTTTTCAGCACTCGA  1380

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Sbjct  1441  TTTTCAGGAAGCCAGGGTCAAAAGCCAAGTTGTCCCGTCGTGACTTCATCAAATCAATCG  1500

Query  1501  GCGGCCTTAAGCCTTTCAAGCAGTTGTGGCGGCGTACTTTTGAGTATGCCACAGTCATTG  1560
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Sbjct  1501  GCGGCCTCAAGCCTTTCAAGCAGTTGTGGCGGCGTACTTTTGAGTATGCCACAGTCATTG  1560

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Query  1741  TCAATGGCTACTGGCTGTCCGACCTGTACTTTAGGCATTCGCGCTGCCAGCATCATTTTG  1800
Sbjct  1741  TCAATGGCTACTGGCTGTCTGACCTGTATTTTAGGCATTCGCGCTGCCAGCATCACTTCG  1800

Query  1801  CTGGCGACATGTCCTCATTCGATTCCACACTTTCTGGGAAAGTGATTGAGTTGGTCAAGG  1860
Sbjct  1801  CTGGCGACATGTCTTCATTCGATTCCACACTTTCTGGGAAAGTGATTGAGTTGGTCAAGG  1860

Query  1861  CGGTTCGCAAGAAAGGGTATGAGCATCATAGAGACCATGATCGCATCTGTAATCTCATTG  1920
Sbjct  1861  CGGTTCGTAAGAAAGGATATGAGCATCACAGAGACCATGATCGCATCTGTAATCTCATTG  1920

Query  1921  ATGTCGCTTACGACCAGCTTGAGCATCAGTTGCTCAACACTACTTCCACTGGCCAGGTCT  1980
Sbjct  1921  ATGTCGCTTACGACCAGCTTGAGCATCAGTTGCTCAACACTACTTCCACTGGCCAGGTCT  1980

Query  1981  ACAAGAAGGGCACTGGACTCACCACCGGTCATTCATCCACCTCTGCGGACAACTCTTTGG  2040
Sbjct  1981  ACAAGAAGGGCACTGGGCTTACCACCGGTCATTCATCCACCTCTGCGGACAATTCGTTGG  2040

Query  2041  GAATAGCCATCCTCTACCTTTTTGCGTGGAAGGAGCTTACTGGCCTCTCGGCCCGTGAGT  2100
Sbjct  2041  GAATAGCCATCCTTTATCTTTTTGCATGGAAGGAGCTCACTGGCCTCTCGGCCCGTGAGT  2100

Query  2101  TCGTTCATTTTAATGAGTTGTCTGATTATGGTGATGATCATGTCCTGTCCTTTCTTGCGA  2160
Sbjct  2101  TCGTTCATTTCAATGAGTTGTCTGACTATGGTGATGATCATGTCCTGTCTTTTCTTGCGA  2160

Query  2161  CCAAACCGGCTGCCTGGAATTTCAAGAACATTCAGAAGGTCATGGCGCGCTGGGGTGTTG  2220
Sbjct  2161  CCAAACCGGCTGCCTGGAATTTCAAGAACATTCAGAAGGTCATGGCGCGCTGGGGTGTTG  2220

Query  2221  AAAATCGTCTCGAAGCAAGTGGGCCTTTGGACTCCATTCCATTCTTGTCCAAGTTTTCCC  2280
Sbjct  2221  AAAATCGCCTTGAAGCAAGCGGGCCTTTGGACTCCATTCCATTCTTGTCCAAGTTTTCCC  2280

Query  2281  GTCGCCTCACTGCCGAAGACCGGGCTGTGTTTGCCAAGTATCAGGTGCCCTTGCCCAAGC  2340
Sbjct  2281  GTCGCCTCACTGCCGAAGACCGGGCTGTGTTTGCCAAGTATCAGGTGCCCTTGCCTAAGC  2340

Query  2341  GGGTTGTCTACCATGACAGGGACCGGTTACTTGGCAAGATGGTTGCTAGGATCAAAAACA  2400
Sbjct  2341  GTGTTGTCTACCATGACAGGGACCGATTACTTGGCAAGATGGTTACTAGGATCAAAAACA  2400

Query  2401  ATGACCCTCGTTACCGGGCAAAAAGGCTGCTTTCTTATCTTAGCCTTACTGCACACCATG  2460
Sbjct  2401  GTGACCCTCGTTACCGGGCAAAAAGGCTGCTTTCTTATCTTAGCCTTACTGCACACCATG  2460

Query  2461  AGGACATCTACAATGGCATCAGCCGTGTGTTGACACGGTCATCCACCATGAAACGGGCAA  2520
Sbjct  2461  AGGACATTTACAATGGCATCAGTCGTGTGTTGACACGGTCATCGACTATGATACGGGCAA  2520

Query  2521  TCAAGCAAATGGGCGTGAGTATCCCAACTTATCAGAAAGTCCTGTCAGACTGGTATCACC  2580
Sbjct  2521  TCAAGCAAATGGGTGTGAGCATCCCGACTTACCAGAAAGTCCTGTCAGACTGGTATCACC  2580

Query  2581  CCACCACACACAGCGTGACCGATGTGTTTGATGAGGTGGCAGGGGAAGCCACTGATGCTG  2640
Sbjct  2581  CCACCACGCACAGTGTGACCGATGTGTTTGATGAAGTGGCAGGGGAAGCCAATGATGCTG  2640

Query  2641  GCCTGGCTTTTTCTTATGGTCAAGTGACATGGGTGGACAGCTTCCTGGGCACCCTTTCAA  2700
Sbjct  2641  GCCTGGCTTTTTCTTATGGCCAAGTAACATGGGTGGACAGCTTCCTGGGGACTCTTTCAA  2700

Query  2701  TGGTTCCTGATTTTGTGAACCCTGCCATCTTCAATTTTGGATATGACAGGCTTTTGCAGA  2760
Sbjct  2701  TGGTTCCTGATTTTGTGAACCCTGCCATCTTTAATTTTGGGTATGACAGGCTTTTGCAGG  2760

Query  2761  TTCAGGCCCGGCGATGGTTGTCATGGCCGATTGAGTTCCTGAGTGCTCAGAATGGTGTTA  2820
Sbjct  2761  TTCAGGCCCGGCGATGGTTGTCATGGCCAATTGAGTTCCTGAGTGCTCAGAATGGTGTTA  2820

Query  2821  GCTCTTCCTCGGAGCTTAGGTCTATGATTGGCAAAAGTTGCTACTCGAGCCTTATCGTGG  2880
Sbjct  2821  GCTCTTCCTCGGAGCTTAGGTCTATGATTGGCAAAAGTTGCTACTCGAGCCTTATCGTGG  2880

Query  2881  ACGTGTTTAGCCAGGCTTTTGAGCCCACTTCAGCTTCCTCCCACCTTGCCAGGCACTGGG  2940
Sbjct  2881  ACGTGTTTAGCCAGGCTTTTGAGCCCACTTCAGCTTCCTCCCACCTTGCCAGGCACTGGG  2940

Query  2941  TTTACCTTTTCTGGTTCTGGTCGTCGAGGTCGGTTTCCCGCTCTTCCTGGCTGAGTGGAA  3000
Sbjct  2941  TTTATCTTTTCTGGTTCTGGTCATCGAGGTCAGCTGCCCGCTCGTCCTGGTTGAGTGGGA  3000

Query  3001  TCACGTACAAGCTGAATCAGGTGCAGTTCACTCTCAATGGCACCATCAATGCAGAGTTTG  3060
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Sbjct  3001  TCACATACAAGCTGAATCAGGTGCAGTTCACTCTTAATGGCACCATCAATGCAGAATTTG  3060

Query  3061  CAGTTATTGGCTGGAAACTTGCTGACTTGTGTGTCATAGCAGCCTGTTCTTTGATTCCGC  3120
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Sbjct  3061  CAGTTATTGGCTGGAAACTTGCTGACTTGTGTGTCATAGCAGCCTGTTCTTTGATTCCGC  3120

Query  3121  TGACACCTTTTCTGGCCCCAGTCTGTGACATCATGCTGCCAAGGATTGATCTATTGATTA  3180
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Sbjct  3121  CGACCCCTTTTCTGGCCCCAATCTGTGACATCATGCTGCCAAGGGTTGATCTGTTGATTA  3180

Query  3181  ACACGATCGTGGGTGTCTTCATTGGGTTGTTCTGGACGAATGTCCCTCCCAATTACAAGG  3240
              |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3181  ACACGATCGTGGGCGTCTTCATTGGGTTGTTCTGGACGAATGTCCCTCCCAATTACAAGG  3240

Query  3241  AAGTGACACATCTCGTTCGACAGCTCCCCTCCATGCGGGGTCCTCTGTTGGTCCAAGCAG  3300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||
Sbjct  3241  AAGTGACACATCTCGTTCGACAGCTCCCCTCCATGCGGGGTCCTCTGTTGGTTCAAGCAG  3300

Query  3301  GTACTGGAACCGGCAAGAGCACTTCCTTCATCAAACACCTGTCATTGGTGGTTGGGGCCC  3360
              | ||||| |||||||| || ||||| |||||||| ||||||||||||||||||||||||
Sbjct  3301  GCACTGGGACCGGCAAAAGTACTTCTTTCATCAAGCACCTGTCATTGGTGGTTGGGGCCC  3360

Query  3361  GTTACAACAAGATTATTGTTATTGAACCCCGATCTGCACTTGTGCGCAGCCTTGTGCCTT  3420
              ||||||| ||||| ||||| |||||||||||||||| |||||||||||||||||||||||
Sbjct  3361  GTTACAATAAGATCATTGTCATTGAACCCCGATCTGCCCTTGTGCGCAGCCTTGTGCCTT  3420

Query  3421  ATGTTAGGGACACTTTGCTGGTTGATGCCACTGGGTGCACTGCCGGATATGATTTTGACC  3480
              |||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||||
Sbjct  3421  ATGTTAGGGACACTCTGCTGGTTGATGCCACTGGCTGCACTGCCGGATATGATTTTGACC  3480

Query  3481  CCACCCGGAAGGTTTGGTATATGACACCGCAGGAGGCTATTTTGCGTCACCGCCACACAT  3540
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Sbjct  3481  CCACCCGGAAGGTTTGGTATATGACACCACAGGAGGCCATTTTGCGTCACCGCCACACAT  3540

Query  3541  TTGACCGTGGCAATCTCATTGTGGTCGATGAGTGCCATTTGGGTGAGGCCGCCTACCGTG  3600
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Sbjct  3541  TCGACCGTGGCAACCTCATCGTGGTCGATGAGTGCCATTTGGGTGAGGCCGCCTACCGTG  3600

Query  3601  TCATACAGCCGTTCCTCAAGTCGCAATCACATTTGGACAGTATTTACCTGACCGCCACTC  3660
              | |||||||||||||||||||||||||||||||||||||| |||||  ||||||||||||
Sbjct  3601  TTATACAGCCGTTCCTCAAGTCGCAATCACATTTGGACAGCATTTATTTGACCGCCACTC  3660

Query  3661  CAAGTGCCTTTAACTTCGAGCAGTGTGAGGCATCAGTCGAACTCAACATAGCCAACGTTT  3720
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3661  CAAGTGCCTTTAACTTCGAGCAGTGTGAGGCATCAGTCGAACTCAACATAGCCAACGTTT  3720

Query  3721  GGCATGTTGCAGTAGATCACCCTGTTTCTCGGGCCGCCGAAGTGGGTGCTTACCTGAAGG  3780
              |||||||||||| |||||||||||||||||||||||||||||||||||||| || ||||
Sbjct  3721  GGCATGTTGCAGTGGATCACCCTGTTTCTCGGGCCGCCGAAGTGGGTGCTTATCTAAAGG  3780

Query  3781  ACTATAGGGCTCTGGTGCTTGACTGCTTACGGGGGCTTCCCAGGATCTCAAAGGTCCTTG  3840
              |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  3781  ACTATAGGGCTCTGGTGCTCGACTGCTTACGGGGGCTTCCCAGGATCTCAAAGGTCCTTG  3840

Query  3841  TTTTCTACCCGTCTAAGGAAGGCGCTGTTTCCTTCGCCGACTCTATAGACAGGCCAGTGT  3900
              |||||||||||| |||||||| ||| ||||||||||||||||||||||||||||||||||
Sbjct  3841  TTTTCTACCCGTCCAAGGAAGGTGCTATTTCCTTCGCCGACTCTATAGACAGGCCAGTGT  3900

Query  3901  CTTTCCTCAACTCTGGTAGCCATGACACCACTGGCAGTGTCATCCTTTCGACAAGCGTAG  3960
              |||| || || ||||| || |||||||||||||||||||||||||||||||||||| || |
Sbjct  3901  CTTTTCTTAATTCTGGCAGTCATGACACCACTGGCAGTGTCATCCTTTCGACAAGTGTGG  3960

Query  3961  CAGATGCCGGGTTGACTATTCCTGCTGTTGATCTGGTGATCTCTCCTTGTTTGGATTACA  4020
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Sbjct  3961  CAGATGCCGGGTTGACTATTCCTGCCGTTGATCTGGTGGTCTCTCCTTGTTTGGATTACA  4020

Query  4021  CAACTACGGGTTTTGGTCTTGAGGTCACTTACGCGTTATTGAACCAAATGCAGATCAAGC  4080
              |||||||||| |||||||||||||||||||| || || ||||||||||||||||||||||
Sbjct  4021  CAACTACGGGCTTTGGTCTTGAGGTCACTTATGCATTGTTGAACCAAATGCAGATCAAGC  4080

Query  4081  AACGCCAGGGGAGAACTGGGCGTACCAATAATGGCCGCTTTGTGTTCCTGCAAGGTCCCA  4140
              |||||||||||||||||| || ||||||||||||||| ||||||||||||||||||||||
Sbjct  4081  AACGCCAGGGGAGAACTGGTCGCACCAATAATGGCCGTTTTGTGTTCCTGCAAGGTCCCA  4140

Query  4141  ACCTCCGTTTACAGACCATGGACTCCTTCCAAGCACTGCCGGAGAATATTGTGACCTCGC  4200
              |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4141  ACCTTCGTTTACAGACCATGGACTCCTTCCAAGCACTGCCGGAGAATATTGTGACCTCGC  4200

Query  4201  TCATGTCCAACGGGGTGACTCTGCCTCAAATTAGGGGCCTTGACCCTGGCCTGGAGGCTC  4260
              |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
Sbjct  4201  TCATGTCCAACGGGGTGACTCTGCCTCAAATCAGGGGCCTTGACCCTGGCCTGGAGGCTC  4260

Query  4261  AGTTTGCCAGGTCTTTCCAGCACTCGACCGGTTTACCGGGCCCCATTGTCGATTCTGCCC  4320
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Sbjct  4261  AGTTCGCCAGGTCTTTCCAGCACTCGACCGGTTTACCTGGCCCTATTGTCGATTCTGCAC  4320

Query  4321  TTGAGAATTTTGACAAGGTGTGGTCAAACTTCTCCTATTGGCGTGAAGGCCGCATGTTGC  4380
              ||||||| |||||||||||||||||||||||| |||||||| ||||||||||||||||||
Sbjct  4321  TTGAGAACTTTGACAAGGTGTGGTCAAACTTCTCTTATTGGCGCGAAGGCCGCATGTTGC  4380

Query  4381  AGTCCATCAATGCCATGGCCTCAGAGGTACAGGAGGTCTTGTTTGATTACAGTGCGGCAG  4440
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Sbjct  4381  AATCCATCAATGCCATGGCCTCAGAAGTGCAGGAGGTCTTGTTTGATCACCTTACGGCAG  4440

Query  4441  GGAACGTCTCTGTTAGTTCCTTCCTGTCAGAAGGGGATGTCCTTGCAGCTTCCAGAGATT  4500
              |||||||||||||| |||||||||||||||||||||||||| |||||||||| ||||
Sbjct  4441  GGAACGTCTCTGTCAGTTCCTTCCTGTCAGAAGGGGATGTCCTCGCAGCTTCCAGGGATT  4500

Query  4501  TCGCAGCCGGTTTGGCTGCCATCAAGTCAGGTTATGAGTGGGATCTCACCGCTTTCGATG  4560
              ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  4501  TCGCAGCCGGTTTGGCTGCCATTAAGTCAGGTTATGAGTGGGATCTCACCGCTTTCGATG  4560

Query  4561  GAGCTCTGACCAAGCTACAATCACTCACTGCAGGTAAAGCCCCGATTGAAGGGGCTCTTC  4620
              |||||||||||||||| |||||||||||||||| ||||||||||||||||||||||||||
Sbjct  4561  GAGCTCTGACCAAGCTGCAATCACTCACTGCAGGGAAAGCCCCGATTGAAGGGGCTCTTC  4620

Query  4621  CTTATGAATATGCAGCAGCGATTGATCAGTCACTCAGATCGGAAG-AGCACACGAATTCA  4679
              |||||||||||||||||||||||||||||||||||||||||||  ||| || ||  ||||||||||
Sbjct  4621  CTTATGAATATGCAGCAGCGATTGATCAGTCACTCAGC-CGGCAGGAG--CACGAATTCA  4677

Query  4680  ATGCAAAATCTTTTTGGCAGGCAACCTGATGATCTTGCATGTGAAAGCCCTCGGGCTAGT  4739
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Sbjct  4678  ATGCAAAATCTTTTTGGCAGGCAACCTGATGATCTTGCGTGTGAAAGCCCTCGGGCTAGT  4737

Query  4740  TCAAAGCCAAAATGGACACCAAGGATATTTTATCTAGAGCCCAAGAGGTTCACACCAACT  4799
              |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  4738  TCAAAGCCAAAATGGACACCAAGGATACTTTATCTAGAGCCCAAGAGGTTCACACCAACT  4797

Query  4800  CTGGAATCAGTTTATTCATTGCTGATTTCAACTTGAAAGACTTGCAAGCCCTTCTCGGGA  4859
              |||||||||||||||||||||||||||||||||||||||||||| |||||||||| |||||||
Sbjct  4798  CTGGAATCAGTTTATTCATTGCTGATTTCAACTTGAAAGATTTGCAAGCCCTCCTCGGGA  4857

Query  4860  ACACTCCTCGCGTTGTAGATGGGGAGTCGAGCGCGGCCTGTCTGCCAGAGACTACCAGCA  4919
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Sbjct  4858  ACACTCCTCGCGGTGCAGATGGGGAATCGAGCGCGGCCTGTCTGCCAGAGACTAGCAGCA  4917

Query  4920  ATGTTAGTCTCGACAGGCAGCAGCATGCGATCGCCTCATCTGCTGTGGGGAAATACACGG  4979
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Sbjct  4918  ATGTTAGTCTCGACAGGCAGCAGCATGCGATCGCCTCATCTGCTGTGGGGAAATACACGG  4977

Query  4980  GTGTTTCTGAGCCAGATTTGACCAGTAGACTTGCCAGGCTGCCTGAATGGTTTCTGCCTC  5039
              ||||||| |||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  4978  GTGTTTCCGAGCCAGATTTGACCAGTAGACTTGCCAGGCTGCCGGAATGGTTTCTGCCTC  5037

Query  5040  GCATTGCACCGGCCGTTTCAAGCGGGTCCAAAGCCCTGCTTGAAACCTCAAGGGCCTCCT  5099
              ||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  5038  GCATTGCACCGGCCGTTTCAAGCGGGTCCAAAGCCCTGCTTGAAGCCTCAAGGGCCTCCT  5097

Query  5100  TCCGGGAGGACCGCGAGAGAGCCAAAGCAGCTGATGACTGTGAGCCTCTGCTCGGTGACA  5159
              | |||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||
Sbjct  5098  TTCGGGAGGACCGCGAGAGAGCCAAAGCAGCTGATGACTGTGGACCTCTGCTCGGTGACA  5157

Query  5160  TCCAGCGGGTTCTAAGTTTCTTATCTTATTTGTGGAAGAGGGCCTTGCACCCCTAATGAA  5219
              ||||||||| |||||||||||||||||||||| |||||||||||||||||||||||
Sbjct  5158  TCCAGCGGGTCCTAAGTTTCTTATCTTATTTGTGGAAAAGGGCCTTGCACCCCTAATGAA  5217

Query  5220  GACTTTGCTCATTCAGACTAGGCCTGAAGATCTTGCATGTGAAAGCCCTCGGGCTAGCCT  5279
              || |||||||||||||| |||||||||||||||||||||||||||||||||||||||||
Sbjct  5218  GATTTTGCTCATTCAGACCAGGCCTGAAGATCTTGCATGTGAAAGCCCTCGGGCTAGCCT  5277

Query  5280  TACACACGATGAGCGCAACCAGCAACAGCCCAGGACGGGCCCCACAATCCGTTCATTCCG  5339
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Sbjct  5278  TACACACGATGAGCGCAACCAGCAACAGCCCAGGACGGGCCCCACAATCTGTTCATTCCG  5337

Query  5340  AACACACCGACGCATTTTTCCTAAAGAAGGAAATTGACTCTCTTGTCAAAGAGCAGTGCA  5399
              |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  5338  AACACACCGACGCATTTTTCCTAAAGAAGGAAATTGACTCTCTTGCCAAAGAGCAGTGCA  5397

Query  5400  ATTTCAAAAGGCAGTCTGGATACTTTATCCGAAAAACCGAGTTTGATGAGCTACAGGCTC  5459
              |||||||| |||||||||| |||||||||||||||||||||||||||| | |||||||
Sbjct  5398  ATTTCAAAAAGCAGTCTGGATATTTTATCCGAAAAACCGAGTTTGATGAGTTGCAGGCTC  5457

Query  5460  GTGTGGACCAGGCCATGATAAATGGCTCTACAACATACGACCCTTCCACCCTCATTGAAA  5519
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Sbjct  5458  GTGTGGACCAGGCCATGATAAATGGCTCTACAACATACGACCCTTCCACCCTCATTGAAA  5517

Query  5520  TGAATCAGCAGCTGACTGACATGACGGCCCGTGCCGAACGGGCCGAACGCATGCTCAGGG  5579
              |||||||||||||||| |||||||||||||| ||||||||||||||||||||||||||||
Sbjct  5518  TGAATCAGCAGCTGACCGACATGACGGCCCGCGCCGAACGGGCCGAACGCATGCTCAGGG  5577

Query  5580  ATGCTGAGGATTCCGCGAAGGAGAATGCCACTTCATTGGCAGATGCTCGAAAGGCCCTCC  5639
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Sbjct  5578  ATGCTGAGGATTCCGCAAAGGAGAATGCCACTTCACTGGTGGATGCCCGAAAGGCCCTCC  5637

Query  5640  ATGAGGCCACAGAGGCAGCCAAGCATGAAAAGACACGCTTGGAGCTTGAGAAGAGTGCAC  5699
              |||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  5638  ATGAGGCCACAGAAGCAGCCAAGCATGAAAAGACGCGCTTGGAGCTTGAGAAGAGTGCAC  5697

Query  5700  TCAAGGGGAAACTTGGGCCCCTTGAGGATTTGCAGAAGCAAGCAAGAAAGCAAGCATCGG  5759
              |||||||||||| |||||||||||||||||||| |||||||||||||||||||||||||| |
Sbjct  5698  TCAAGGGGAAACTCGGGCCCCTTGAGGATTTGCAAAAGCAAGCAAGAAAGCAAGCATCCG  5757

Query  5760  ACCCTGAGGAGGTTGCTGCTCGCACTCGCTTGATAACGGAGCTTCAAAGCCAGCTCGCCG  5819
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Sbjct  5758  ACCCTGAGGAAGTCGCTGCTCGCACTCGATTGATAACGGAGCTTCAAAGCCAGCTCGCCG  5817

Query  5820  CTGTTAATGCAGCTTTTGCTGCTGAAACAGCCAAAGTCCGGACCTCCCATGCGAAGGTCA  5879
Sbjct  5818  CTGTCAATGCAGCTTTTGCTGCTGAAACAGCCAAAGTCCGGACCTCTCATGCGAAGGTTA  5877

Query  5880  CCGAACTTGAAGCATCCTTGAACTGCGAAAGGCTCAAGGCCGAACGTGCCCAGAAGGCAG  5939
Sbjct  5878  CCGAGCTTGAAGCATCTTTGAACTGCGAAAGGCTCAAGGCCGAACGTGCCCAAAAGGCAG  5937

Query  5940  TGTTCAACCCTAAGCCTATCATTGCAGATGAGCCTCTTAAGCCTCTAGAACCCTTTGAGG  5999
Sbjct  5938  TGTTCAACCCTAAGCCCATTATTGCAGATGAGCCTCTCAAGCCTCTAGAACCCTTTGAGG  5997

Query  6000  CAGATCTCGCCAAGGACGGTGCTGTTTTACGCAGAGTTTTAAGCACCGAATCGCTTGCCC  6059
Sbjct  5998  CAGATCTCGCCAAGGACGGTGCTGTTTTACGCAGAGTTTTAAGCACCGAATCGCTTGCCC  6057

Query  6060  TACTGCACAAGTGTGCAGAGGTGCGCAGAGAGAACACCCGGGAGTATGCATACTCCCTGC  6119
Sbjct  6058  TACTGCACAAATGTGCAGAGGTGCGCAGAGAGAACACCCGGGAGTATGCATACTCCCTGC  6117

Query  6120  TCGAAGTGGCCAAGCGACGAGATCCCCGCAATCACGTGGGTTATCGTGATTATGCGAAGA  6179
Sbjct  6118  TCGAAGTGGCCAAGCGACGAGATCCCCGCAATCACGTGGGTTATCGTGATTATGCGAAGA  6177

Query  6180  TGGTTGTTCAGAAGATCAAGTCAACTTCCGCCAGGAAGCGCGCCAAGTATCAGCCGTTAC  6239
Sbjct  6178  TGGTTGTCCAGAAGATCAAGTCAACTTCCGCTAGGAAGCGCGCCAAGTATCAGCCGTTAC  6237

Query  6240  TTGATTCGCTTTATGATTCCATGGCTTTTGAGAACACTCTCAAGCTTAAGGAGATCAAAG  6299
Sbjct  6238  TTGATTCGCTTTATGAGTCCATGGCTTTTGAGAACACTCTCAAGCTCAAGGAGATCAAAG  6297

Query  6300  CTACCTACCGCACACTTCTGGAACCTCAGAGCAAAGAATCTGAGGCTAATTCTGAGAAGG  6359
Sbjct  6298  CCACCTACCGCACACTTCTGGAACCTCAGAGCAAAGAATCTGAGGCTAATTCTGAGAAAG  6357

Query  6360  TGCAGGCCGGTGCTGGCAGCTGGTGGGATGACCTCCTATATGACGCCAAACTTCTTTTCC  6419
Sbjct  6358  TGCAGGCCGGTGCTGGCAGCTGGTGGGATGACCTCTTATATGACGCCAAACTTCTTTTCC  6417

Query  6420  GTCGTGCCAAGGTGGAATTCCTTGTCAGGACGCAAAATCCTGAGACGACTGGTTGGTTTT  6479
Sbjct  6418  GTCGTGCCAAGGTGGAATTCCTTGTCAGGACCCAAAATCCTGAGACGACTGGTTGGTTTT  6477

Query  6480  CGTCCCTGCGAGCGAAAACCTGGGCTTTTTGTGGTAAATTAAAATCCATACTGCCCTTTT  6539
Sbjct  6478  CGTCCCTGCGAGCGAAAACCTGGGCTTTTTGTGGTAAATTAAAATCCATACTGCCCTTTT  6537

Query  6540  CTTGGTTCTCCTCCCCCACTCCAACAACAAGCGCTTAACTCCCAAAATAAGGGGTTGAGC  6599
Sbjct  6538  CTTGGTTCTCCCCCCCCATTCCGGCAACAAACGCCTAACTCCTAAAACAAGGGGTTGAGC  6597

Query  6600  ATAGGGGGTCTTAGAGGCCAAATG  6623
Sbjct  6598  ACAGGGGGTCTTAGAGGCCAAATG  6621
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Range 2: 5240 to 5274

| Score | Expect | Identities | Gaps | Strand | Frame |
|---|---|---|---|---|---|
| 60.2 bits(32) | 4e-11() | 34/35(97%) | 0/35(0%) | Plus/Plus | |

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Query  4704  CCTGATGATCTTGCATGTGAAAGCCCTCGGGCTAG  4738
Sbjct  5240  CCTGAAGATCTTGCATGTGAAAGCCCTCGGGCTAG  5274
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Fusarium graminearum dsRNA mycovirus-1 clone pDK-2 sequence

Sequence ID: **AF443212.1** Length: 1080 Number of Matches: 1

Range 1: 2 to 1080

| Score | Expect | Identities | Gaps | Strand | Frame |
|---|---|---|---|---|---|
| 1712 bits(927) | 0.0() | 1031/1082(95%) | 4/1082(0%) | Plus/Plus | |

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Query  3751  GGGCCGCCGAAGTGGGTGCTTACCTGAAGGACTATAGGGCTCTGGTGCTTGACTGCTTAC  3810
Sbjct  2     GGGCCGCCGAAGTGGGTGCTTATCTAAAGGACTATAGGGCTCTGGTGCTCGACTGCTTAC  61

Query  3811  GGGGGCTTCCCAGGATCTCAAAGGTCCTTGTTTTCTACCCGTCTAAGGAAGGCGCTGTTT  3870
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Sbjct  62    GGGGGCTTCCCAGGATCTCAAAGGTCCTTGTTTTCTACCCGTCCAAGGAAGGTGCTGTTT  121

Query  3871  CCTTCGCCGACTCTATAGACAGGCCAGTGTCTTTCCTCAACTCTGGTAGCCATGACACCA  3930
              ||||||||||||||||||||||||||||||||||  || ||  |||||  || ||||||||||
Sbjct  122   CCTTCGCCGACTCTATAGACAGGCCAGTGTCTTTTCTTAATTCTGGCAGTCATGACACCA  181

Query  3931  CTGGCAGTGTCATCCTTTCGACAAGCGTAGCAGATGCCGGGTTGACTATTCCTGCTGTTG  3990
              |||||||||||||||||||||||||  ||  |||||||||||||||||||||||||| ||||
Sbjct  182   CTGGCAGTGTCATCCTTTCGACAAGTGTGGCAGATGCCGGGTTGACTATTCCTGCCGTTG  241

Query  3991  ATCTGGTGATCTCTCCTTGTTTGGATTACACAACTACGGGTTTTGGTCTTGAGGTCACTT  4050
              ||||||||  |||||||||||||||||||||||||||||||  ||||||||||||||||||
Sbjct  242   ATCTGGTGGTCTCTCCTTGTTTGGATTACACAACTACGGGCTTTGGTCTTGAGGTCACTT  301

Query  4051  ACGCGTTATTGAACCAAATGCAGATCAAGCAACGCCAGGGGAGAACTGGGCGTACCAATA  4110
              | ||  || ||||||||||||||||||||||||||||||||||||||||||  ||  |||||||
Sbjct  302   ATGCATTGTTGAACCAAATGCAGATCAAGCAACGCCAGGGGAGAACTGGTCGCACCAATA  361

Query  4111  ATGGCCGCTTTGTGTTCCTGCAAGGTCCCAACCTCCGTTTACAGACCATGGACTCCTTCC  4170
              |||||||  |||||||||||||||||||||||||||  ||||||||||||||||||||||||||
Sbjct  362   ATGGCCGTTTTGTGTTCCTGCAAGGTCCCAACCTTCGTTTACAGACCATGGACTCCTTCC  421

Query  4171  AAGCACTGCCGGAGAATATTGTGACCTCGCTCATGTCCAACGGGGTGACTCTGCCTCAAA  4230
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422   AAGCACTGCCGGAGAATATTGTGACCTCGCTCATGTCCAACGGGGTGACTCTGCCTCAAA  481

Query  4231  TTAGGGGCCTTGACCCTGGCCTGGAGGCTCAGTTTGCCAGGTCTTTCCAGCACTCGACCG  4290
              | |||||||||||||||||||||||||||||||  |||||||||||||||||||||||||
Sbjct  482   TCAGGGGCCTTGACCCTGGCCTGGAGGCTCAGTTCGCCAGGTCTTTCCAGCACTCGACCG  541

Query  4291  GTTTACCGGGCCCCATTGTCGATTCTGCCCTTGAGAATTTTGACAAGGTGTGGTCAAACT  4350
              |||||||  |||||  ||||||||||||||  ||||||||  |||||||||||||||||||||||
Sbjct  542   GTTTACCTGGCCCTATTGTCGATTCTGCACTTGAGAACTTTGACAAGGTGTGGTCAAACT  601

Query  4351  TCTCCTATTGGCGTGAAGGCCGCATGTTGCAGTCCATCAATGCCATGGCCTCAGAGGTAC  4410
              ||||  ||||||||  ||||||||||||||||||  ||||||||||||||||||||||  ||  |
Sbjct  602   TCTCTTATTGGCGCGAAGGCCGCATGTTGCAATCCATCAATGCCATGGCCTCAGAAGTGC  661

Query  4411  AGGAGGTCTTGTTTGATTACAGTGCGGCAGGGAACGTCTCTGTTAGTTCCTTCCTGTCAG  4470
              ||||||||||||||||  ||  ||||||||||||||||||||  ||||||||||||||||
Sbjct  662   AGGAGGTCTTGTTTGATCACCTTGCGGCAGGGAACGTCTCTGTCAGTTCCTTCCTGTCAG  721

Query  4471  AAGGGGATGTCCTTGCAGCTTCCAGAGATTTCGCAGCCGGTTTGGCTGCCATCAAGTCAG  4530
              ||||||||||||| ||||||||||| ||||||||||||||||||||||||||| |||||||
Sbjct  722   AAGGGGATGTCCTCGCAGCTTCCAGGGATTTCGCAGCCGGTTTGGCTGCCATTAAGTCAG  781

Query  4531  GTTATGAGTGGGATCTCACCGCTTTCGATGGAGCTCTGACCAAGCTACAATCACTCACTG  4590
              ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  782   GTTATGAGTGGGATCTCACCGCTTTCGATGGAGCTCTGACCAAGCTGCAATCACTCACTG  841

Query  4591  CAGGTAAAGCCCCGATTGAAGGGGCTCTTCCTTATGAATATGCAGCAGCGATTGATCAGT  4650
              ||||  ||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   CAGGGAAAGCCCCGATTGACGGGGCTCTTCCTTATGAATATGCAGCAGCGATTGATCAGT  901

Query  4651  CACTCAGATCGG-AAGAGCACACGAATTCAATGCAAAATCTTTTTGGCAGGCAACCTGAT  4709
              |||||||  ||| | |||  |||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   CACTCAG-CCGGCAGGAG--CACGAATTCAATGCAAAATCTTTTTGGCAGGCAACCTGAT  958

Query  4710  GATCTTGCATGTGAAAGCCCTCGGGCTAGTTCAAAGCCAAAATGGACACCAAGGATATTT  4769
              |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  959   GATCTTGCGTGTGAAAGCCCTCGGGCTAGTTCAAAGCCAAAATGGACACCAAGGATACTT  1018

Query  4770  TATCTAGAGCCCAAGAGGTTCACACCAACTCTGGAATCAGTTTATTCATTGCTGATTTCA  4829
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1019  TATCTAGAGCCCAAGAGGTTCACACCAACTCTGGAATCAGTTTATTCATTGCTGATTTCA  1078

Query  4830  AC  4831
              ||
Sbjct  1079  AC  1080
```

Fusarium graminearum dsRNA mycovirus-1 clone pDK-1 sequence

Sequence ID: **AF443213.1** Length: 825 Number of Matches: 1

Range 1: 1 to 825

| Score | Expect | Identities | Gaps | Strand | Frame |
|---|---|---|---|---|---|
| 1310 bits(709) | 0.0() | 788/826(95%) | 5/826(0%) | Plus/Plus | |

```
Query  1191  ACGTATACCGATGAGTTGTTGGATGACCTGAGACT-C--CAGGCCGTTGAATTCCGACGC  1247
              |||||  |||||||||  ||||||||||||||||||  |   ||  ||||  |||  |||||||||
Sbjct  1      ACGTACACCGATGAGCTGTTGGATGACCTGAGACTTCTTCATGCCG-TGAGTTCCGACGC  59

Query  1248  ACTGAGGAGTATGCCAGCCTCGACAACGAGTTGAAATCAACCTCACGCTACTTCAGGTCG  1307
              |||||||||||||||||||||||||||  |||||||  ||||||||||||||||  |||||||||
Sbjct  60    ACTGAGGAGTATGCCAGCCTCGACAATGAGTTGAGATCAACCTCACGCTATTTCAGGTCG  119
```

```
Query  1308  CCTCGTTACGATTTTCCTGATTTGGAACTGGACGACGTGTGGTTTTTGGTCAAGGATACT  1367
             ||  ||||||||||| ||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  120   CCCCGTTACGATTTCCCTGATTTGGAACTGGACGACGTGTGGTTTTTGGTCAAGGACACT  179

Query  1368  TTTCAGCACTCGAAGTTGACCCCCTTCAACTACATCATTCAGATGTGGGAGAAGAAGTAT  1427
             ||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
Sbjct  180   TTTCAGCACTCGAAGTTGACCCCCTTCAACTACATCATCCAGATGTGGGAGAAGAAGTAT  239

Query  1428  GGCCTGGGTGCCTTTTTCAGGAAGCCTGGGTCAAAGGCCAAATTGTCTCGTCGTGACTTC  1487
             |||||||||||||||||||||||||| |||||||| ||||| ||||| ||||||||||||
Sbjct  240   GGCCTGGGTGCCTTTTTCAGGAAGCCAGGGTCAAAAGCCAAGTTGTCCCGTCGTGACTTC  299

Query  1488  ATCAAGTCAATCGGCGGCCTTAAGCCTTTCAAGCAGTTGTGGCGGCGTACTTTTGAGTAT  1547
             |||||  |||||||||||||| |||||||||||||||||||||||||||| ||||||||
Sbjct  300   ATCAAATCAATCGGCGGCCTCAAGCCTTTCAAGCAGTTGTGGCGGCGTACTCTTGAGTAT  359

Query  1548  GCCACAGTCATTGTGCCTGTGTCGGCAGTGAGTGTTAAGGGCGAAGCCCTCCCTCCCAAG  1607
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360   GCCACAGTCATTGTGCCTGTGTCGGCAGTGAGTGTTAAGGGCGAAGCCCTCCCTCCCAAG  419

Query  1608  AAATGG-ATGGAGGACAAAGTGCGTACTATTATTGGCACGCCGCTTGTCCATTACATAAT  1666
             ||||||  |||||||||||||||||||||||  |||||  ||||||||||  ||||||||||
Sbjct  420   AAATGGAATGGAGGACAAAGTGCGTACTATCATTGGTACGCCGCTTGTTCATTACATAAT  479

Query  1667  GACCACCATTTGGAATTACGAACCCAATCATCGTTTTGCTTGGGTGAGCACTCCTACCAA  1726
             ||||||||||||||  ||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  480   GACCACCATTTGGAACTACGAACCCAACCATCGTTTTGCTTGGGTGAGCACTCCTACCAA  539

Query  1727  AATTGGCATGCCACTCAATGGCTACTGGCTGTCCGACCTGTACTTTAGGCATTCGCGCTG  1786
             |||||||||||||||||||||||||||||||||||||| ||||||||  ||||||||||||||||
Sbjct  540   AATTGGCATGCCACTCAATGGCTACTGGCTGTCTGACCTGTATTTTAGGCATTCGCGCTG  599

Query  1787  CCAGCATCATTTTGCTGGCGACATGTCCTCATTCGATTCCACACTTTCTGGGAAAGTGAT  1846
             ||||||||  ||  ||||||||||||||  ||||||||||||||||||||||||||||||||
Sbjct  600   CCAGCATCACTTCGCTGGCGACATGTCTTCATTCGATTCCACACTTTCTGGGAAAGTGAT  659

Query  1847  TGAGTTGGTCAAGGCGGTTCGCAAGAAAGGGTATGAGCATCATAGAGACCATGATCGCAT  1906
             ||||||||||||||||||||  ||||||||  ||||||||||  ||||||||||||||||||
Sbjct  660   TGAGTTGGTCAAGGCGGTTCGTAAGAAAGGATATGAGCATCACAGAGACCATGATCGCAT  719

Query  1907  CTGTAATCTCATTGATGTCGCTTACGACCAGCTTGAGCATCAGTTGCTCAACACTACTTC  1966
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720   CTGTAATCTCATTGATGTCGCTTACGACCAGCTTGAGCATCAGTTGCTCAACACTACTTC  779

Query  1967  CACTGGCCAGGTCTACAAGAAGGGCACTGGACTCACCACCGGTCAT  2012
             |||||||||||||||||||||||||||||||  ||  ||||||||||||
Sbjct  780   CACTGGCCAGGTCTACAAGAAGGGCACTGGGCTTACCACCGGTCAT  825
```

## Taxonomy

### Reports

### Lineage

| Organism | Blast Name | Score | Number of Hits | Description |
|---|---|---|---|---|
| Fusarium graminearum dsRNA mycovirus-1 | viruses | 10739 | 3 | Fusarium graminearum dsRNA mycovirus-1 hits |

### Organism

| Description | Score | E value | Accession |
|---|---|---|---|
| Fusarium graminearum dsRNA mycovirus-1 [viruses ] | | | |
| Fusarium graminearum dsRNA mycovirus-1 strain DK-21, complete genome | 10739 | 0.0 | AY533037 |
| Fusarium graminearum dsRNA mycovirus-1 clone pDK-2 sequence | 1712 | 0.0 | AF443212 |
| Fusarium graminearum dsRNA mycovirus-1 clone pDK-1 sequence | 1310 | 0.0 | AF443213 |

### Taxonomy

| Taxonomy | Number of hits | Number of Organisms | Description |
|---|---|---|---|
| Fusarium graminearum dsRNA mycovirus-1 | 3 | 1 | Fusarium graminearum dsRNA mycovirus-1 hits |

**Supplementary table S3.1** DON content in harvested wheat seeds after infection with F. graminearum strains having or not having FgV1 (Fg-4-1 and Fg-4-2). Water inoculated plants were used as control to measure the DON content in harvested seeds.

| Fg-4-1 | | | Fg-4-2 | | | Water | | |
|---|---|---|---|---|---|---|---|---|
| | DON | | | DON | | | DON | |
| Weight (g) | (ng/sample) | (ppm) | Weight (g) | (ng/sample) | (ppm) | Weight (g) | (ng/sample) | (ppm) |
| 0.07832 | 485.2 | 6.2 | 0.50688 | 0 | 0 | 0.97177 | 0 | 0 |
| 0.57347 | 619.7 | 1.1 | 0.65791 | 0 | 0 | 0.83739 | 0 | 0 |
| 0.14619 | 0 | 0 | 0.59894 | 0 | 0 | 0.93269 | 0 | 0 |
| 0.08085 | 27.4 | 0.34 | 0.59244 | 0 | 0 | 1.04553 | 0 | 0 |
| 0.00434 | 6.5 | 1.5 | 0.68239 | 0 | 0 | 1.34753 | 0 | 0 |
| 0.3283 | 3666.1 | 11.2 | 0.63993 | 0 | 0 | 1.04088 | 0 | 0 |
| 0.23895 | 4434.2 | 18.6 | 0.46087 | 0 | 0 | 1.21904 | 0 | 0 |
| 0.13557 | 1268.5 | 9.4 | 0.61796 | 0 | 0 | 1.01127 | 0 | 0 |
| 0.00284 | 602.9 | 212.3 | 0.5485 | 0 | 0 | 0.86737 | 0 | 0 |
| 0.15108 | 34.8 | 0.23 | 0.69121 | 0 | 0 | 1.2794 | 0 | 0 |
| 0.00685 | 7.8 | 1.1 | 0.70713 | 0 | 0 | 0.9816 | 0 | 0 |
| 0.0089 | 0 | 0 | 0.42893 | 0 | 0 | 0.6652 | 0 | 0 |
| 0.00555 | 0 | 0 | 0.50902 | 0 | 0 | 1.13615 | 0 | 0 |
| 0.00538 | 0 | 0 | 0.54454 | 0 | 0 | 1.13051 | 0 | 0 |
| 0.00389 | 0 | 0 | 0.68499 | 0 | 0 | 0.75206 | 0 | 0 |
| Average | 761.99 | 17.46 | | 0 | 0 | | 0 | 0 |